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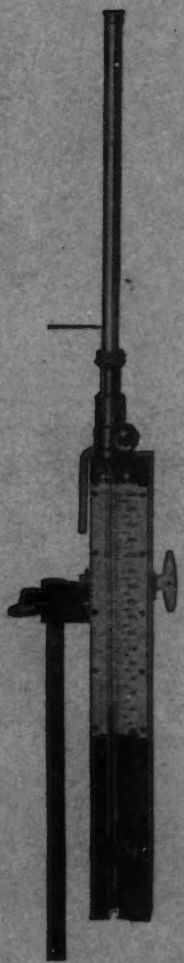

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CHARACTER OF THE BLOOD-CLOT IN THE THYROIDECTOMIZED ADULT OPOSSUM

MAMIE GRAY

From The University of Texas,[1] Austin

Received for publication November 26, 1923

In the March, 1923, number of Endocrinology, Madame Parhon reports the lowered calcium content in the blood of thyroidectomized sheep. In this connection the following observation on the character of the blood-clot after thyroidectomy is of interest. The observation was made incidental to a study of coagulation time of the blood of opossum pouch young compared with that of the adult.

The method employed, where an ample blood sample could be secured, was that of Cannon and Mendenhall (1), which has the advantage of yielding a permanent record of each experiment and is furthermore simple of operation. I have introduced a modification in the form of a cannula which serves both for drawing the sample of blood and for holding the blood during the test. A glass tube is drawn out at one end to the desired uniform diameter with the last centimeter bent at an angle of 45°. The tip is then further drawn out to a short capillary tube for puncturing the blood vessel. The blood is drawn into the (paraffined) tube by suction. It is thought that this device makes for cleanliness and convenience.

With this method the coagulation time of adult blood was found to be as follows:

Animals	*Time*
Normal adults	
Without ether	5 minutes, 19 seconds
Etherized	6 minutes, 26 seconds
Thyroidectomized adults	
Without ether	5 minutes, 4 seconds
Etherized	7 minutes, 25 seconds

[1] Contribution from the Department of Zoölogy, University of Texas, no. 166.

The coagulation time for the pouch young was as follows:

Weight of embryos	*Time*
0.2 to 0.7 gram	6 minutes, 7 seconds
0.7 to 1.0 gram	6 minutes, 5 seconds
1.0 to 6.0 grams	5 minutes, 17 seconds
8.0 to 20.0 grams	5 minutes, 42 seconds
120.0 to 160.0 grams	3 minutes, 54 seconds

Except for the last figures, the explanation of which I shall not go into here, the differences in time between the pouch young and adults and between normal and thyroidectomized adults are of no great significance. The chief interest, however, lies in the character of the clot. Normal adult blood produces a firm clot which in our apparatus fully supports the writing lever the instant the clot forms; hence the end point is sharp and definite, as seen in A and B, figure 1. The blood-clot of the pouch young, however, is thin and only inadequately supports the plunger of the writing lever. Typical records secured from embryonic blood are shown in C and D, figure 1. The difference in the two series is apparent.

Emmel, Levinson and Fisch (2) noted the same phenomenon in the blood of fetal pigs, where the blood-clot "is seldom of sufficient firmness to permit a complete inversion of the tube." The authors, who used the test-tube method of determining the end point, describe the fetal blood-clot as a "sliding clot."

Having on hand at the time four adult opossums, male and females, from which the thryoids had been removed four months previously by some of Prof. Carl Hartman's students, I used these animals also for my experiments and found the blood-clot to be of the same consistency as that of the pouch young. Two typical records are shown in E and F, figure 1. They are indistinguishable from the records secured from the blood of pouch young.

At least two samples were taken from each of the four animals and all consistently gave the same character of record.

These results differ from those of Lidsky and Kottmann (4), which were made chiefly on clinical material. In cases of hyperthyroidism these authors found a *lengthening* of coagulation time and a *thinning* of the clot ("Gerinnungsverzögerung" und "eine auffallende Schwäche der Koagula"). The same occurred in a rabbit and a dog receiving intravenous injection of thyroid extract. In cases of hypothyroidism (myxedema, goiter, etc.), on the other hand, there was a *shortening* of the coagulation time and a much *thicker* clot. However, in one tetany case, presumably with parathyroid involvement, the *shortening* of coagulation time was associated with a *firmer* clot. In correlation with these observations Kottmann and Lidsky find the blood of Basedow cases to have a fibrin content of 0.16 per cent and myxedematous patients of 0.39 per cent, as compared with the normal 0.226 per cent.

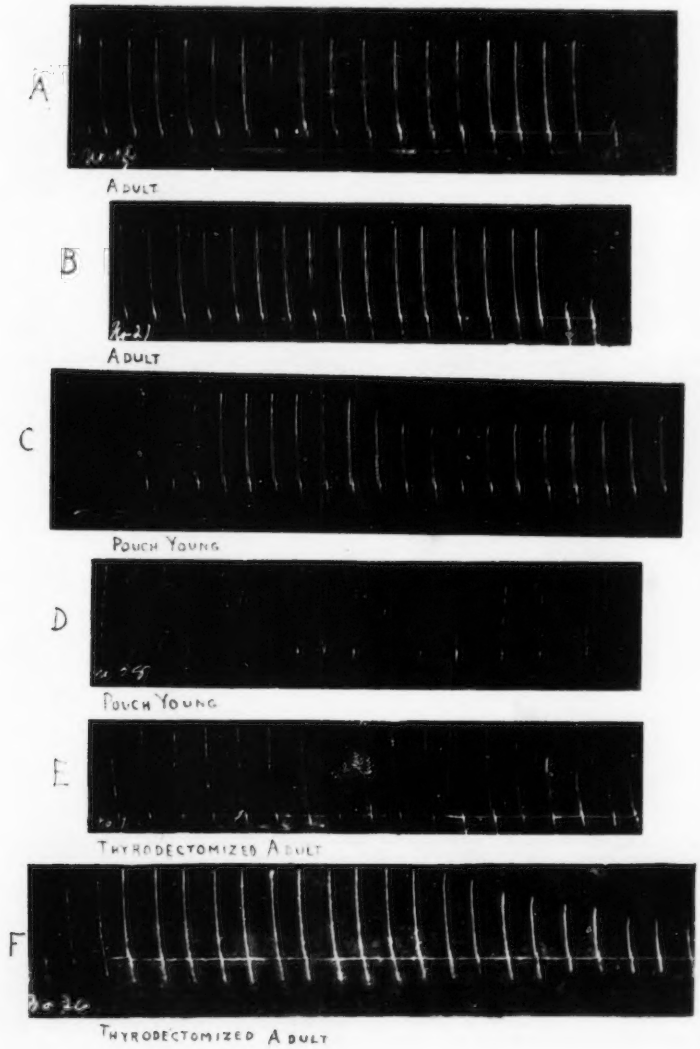

A
ADULT
B
ADULT
C
POUCH YOUNG
D
POUCH YOUNG
E
THYRODECTOMIZED ADULT
F
THYRODECTOMIZED ADULT

Fig. 1

In contrast to these results our thyroidectomized opossums, four months after operation, possessed blood which consistently refused to form a firm clot, although the coagulation time was little changed.

The thin clot of embryonic blood Emmel and his students attribute to the small amount of fibrin, which they find to be one-eighth that of the adult. The calcium content they find to be identical in embryo and adult, although in the former the calcium is probably partly in the non-dissociated form, for calcium ions hasten coagulation.

Finally, Biedl quotes Albertoni to the effect that thyro-parathyroidectomy effects an increase of fibrin from 2.51 per cent to 4.69 per cent.

The above-described experiments on the opossum blood, though at variance with these studies on man and dog, concern clear-cut thyroidectomy without parathyroid involvement. For in this animal the parathyroids of the adult are not imbedded in the thyroids nor near them; in fact, it is not known where in the body they are situated. Hence any disturbance of the parathyroids is absolutely eliminated. The opossum, therefore, in contrast to dog, cat or rabbit, would seem an ideal animal for the study of pure thyroidectomy effects.

BIBLIOGRAPHY

(1) Cannon and Mendenhall: This Journal, 1914, xxxiv, 225.
(2) Emmel, Levinson and Fisch: Journ. Exper. Med., 1920, xxxi, 177.
(3) Hatai: Journ. Biol. Chem., 1918, xxxv, 527.
(4) Kottmann and Lidsky: Zeitschr. f. Klin. Med., 1910, lxxi, 344, 362.
(5) Parhon: Endocrinol. 1923, vii, 311.
(6) Toyama: Journ. Biol. Chem., 1919, xxxviii.

KINEMATOGRAPHIC METHODS IN THE STUDY OF CAPILLARY CIRCULATION

AUGUST KROGH AND P. BRANDT REHBERG

From the Laboratory of Zoöphysiology, University of Copenhagen

Received for publication November 27, 1923

In studying the physiology of capillaries the microscope is the chief instrument of research, and the general method is to observe directly the "spontaneous" changes taking place and the reactions to various experimental procedures. This method is open to the objection that it does not furnish any documentary evidence of the observations reported, and it has the further and much more serious drawback that several capillary reactions take place at such a rate that it is exceedingly difficult to observe them correctly. An important improvement in technique can therefore be effected by photographing typical reactions on the kinema film.

The problem of obtaining such photographic records presents several difficulties, and it will probably be useful, therefore, to describe in some detail the methods which we have so far evolved and which allow us to obtain pictures of the circulation in transparent tissues of the frog at rates varying from 20 photographs per second downwards.

We have used the arrangement on an optical bench provided by the firm of Leitz for micro-projection. The source of light is a 5 amp. arc-lamp with horizontal positive carbon. A special lens which will stand the heating is placed very near the arc and provides a parallel beam of light. This light is filtered through a suitable solution in a 3 to 5 cm. glass-trough. The filtration is a very important point as the capillaries in many tissues are very senitive to light. After numerous trials we use a solution of copper sulphate and picric acid which we adjust by means of a spectroscope, so as to cut off as completely as possible both the red and the violet end of the spectrum. We have noticed that the short wave-lengths are almost as harmful to our objects as the heat rays. For visual observation before the photographing we put an ordinary 20 candle incandescent lamp in front of the arc-lamp. As the object to be photographed must be horizontal, the microscope is arranged on the bench in the upright position behind a screen which allows only the illuminating beam of light to reach the mirror. For the lowest magnification the concave mirror will give sufficient illumination, while the objectives of shorter focal length are used in combination with the plane mirror and special condensers. The iris diaphragm combined with the

condenser regulates the illumination and a diaphragm at the level of the table cuts off all light beyond an area slighly in excess of that covered by the objective. It is important to protect the tissues by admitting no more light than is necessary. We use a projection eye-piece (Leitz no. 2) adjusted for a distance of 200 mm. The camera is a small Ernemann taking 30 m. film. It has been arranged for turning by motor at rates which can

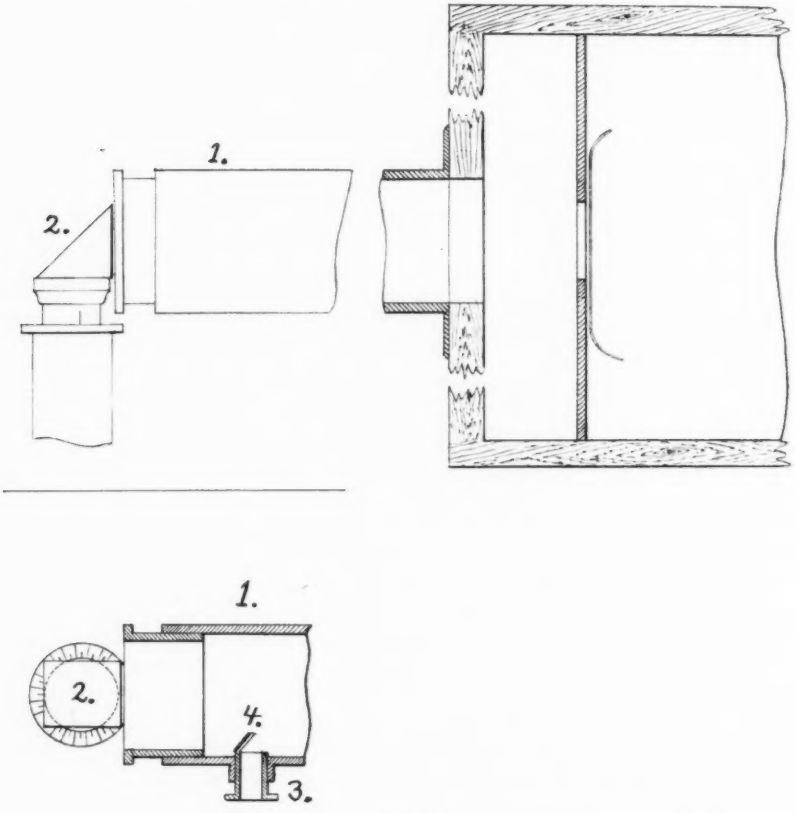


Fig. 1

be regulated within wide limits. It is rigidly mounted on supports projecting from the wall and quite distinct from those carrying the optical bench, so as to prevent the unavoidable slight vibration of the camera from affecting the microscope. The objective of the camera is of course removed and instead of it we have the tube *1* (fig. 1) carrying a diaphragm with a hole of 6 mm. just in front of the reflecting prism *2*, which is placed on top of the

microscope. The tube is provided with a side-tube *3*, through which the image projected on the film can be directly observed by means of the mirror, *4*. A magnifying lens can be arranged in the side tube, if necessary. This arrangement originally described by Neuhauss (1) greatly facilitates the focusing of the image, and the reaction is watched throughout and adjustments made if necessary. With some practice it becomes possible to estimate whether the illumination is suitable and to regulate it accordingly.

The microscope is fitted with an attachable mechanical stage carrying a thin brass plate sliding on the fixed stage. The animal which must of course be narcotized or curarized is put on this plate and the part to be photographed is arranged on a cover slip cemented over a suitable hole in the plate.

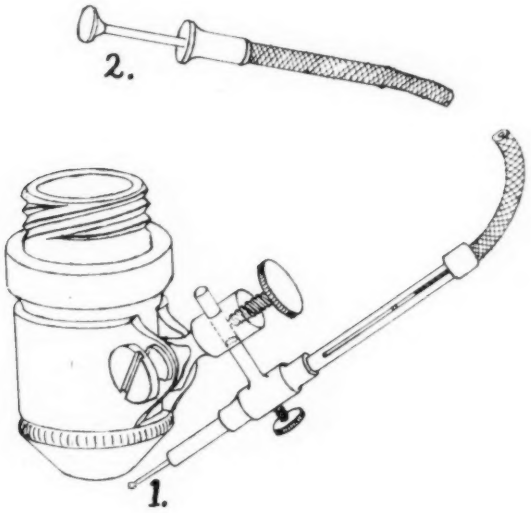


Fig. 2

To stimulate locally the tissues or vessels photographed we use the attachment, shown in figure 2, which is rigidly fixed to a special objective (Reichert no. 3, 17.9 mm. focal distance) and which can be adjusted so that the needle *1*, on pressing the piston *2*, will be pushed on to the center of the field and at a distance from the lens corresponding as closely as may be desired to the focal distance. For mechanical stimulation we use a metal needle and adjust it so as to penetrate that layer of tissue which is sharply focused. For the placing of small drops of reagents we use a glass needle with a knob of $\frac{1}{2}$ mm. diameter and adjusted so that its lower surface will just touch the tissue when it is pushed home.

The details of our methods—in so far as they require any special reference —are best brought out in a short description of a series of pictures selected for the illustration of normal capillary circulation and typical capillary reactions.[1]

For the first of these the tongue of a frog has been pinned out with the lower surface upwards and photographed by means of a low power lens. (Seibert 00 without eyepiece. Magnification 10 diameters.) At such low magnification the depth of focus is a disturbing factor and in the picture, which shows the pulsation of the main artery of the tongue, the bands of striped muscle running across the vessels have a somewhat disturbing effect. On the whole our experience points to the conclusion that in microkinematography the best results are obtained at high magnifications.

For a second series of pictures the bladder of a frog was made to protrude outside the body wall and was distended by the injection of saline. A plane horizontal surface was secured by a cover glass supported on a lump of modelling wax. The pictures taken at 3 different magnifications with objectives 00 Leitz apochromates 16 and 8 mm. show very distinctly the penetration of the pulse wave through the capillaries and its annihilation by interference in the venules.

Corresponding pictures have been taken of the frog's lung showing at a low magnification how a very large number of capillary channels are given off directly from a comparatively large artery, and illustrating the enormous surface development of the circulation in the alveoli. The picture taken of the pulmonary circulation at a high magnification (90 diameters) gives very striking evidence of the peculiar elastic properties of the red corpuscles (2). In several places and especially at one point somewhat above the center of the picture the cellular islands separating the capillary channels project with a sharp edge against the current. In such places the red corpuscles are often caught and one such case is shown in the drawing, figure 3. This corpuscle remains hanging on the edge for about 4 seconds. At first it is riding nearly along the short axis and both ends are bent down in the direction of the current. Somewhat later it slides along toward one of the ends and becomes rather sharply bent into the shape of a large and a small sac. Finally, when the current becomes slack in the interval between the two beats of the heart, the corpuscle slides off the edge. The four lower figures show four consecutive stages of this process with an interval of 0.06 second.

[1] It is well known to teachers in physiology that it is by no means easy to convey to the students correct ideas regarding the circulation of the blood in microscopic vessels, partly on account of the difficulties inseparable from demonstrations by means of microscopes or direct microprojection. One of the objects of the film here described is to supply material suitable for teaching the main facts of the circulation through the tissues, and it has been arranged therefore that copies can be obtained from the Laboratory of Zoöphysiology, 11 Ny Vestergade, Copenhagen.—The total length of the film is 225 m. and the price has been fixed at 300 Kroner Danish.

In the last figure, about 0.3 second after the release, the shape has practically returned to normal, in consequence of the elastic properties of the corpuscle. When the riding corpuscle is watched on the kinema film, the current appears to be so rapid that it is not very surprising to see such effects, but it must be remembered that the rate of flow is magnified in the same proportion as the objects. On the film the actual rate can be approximately measured by noting the shifting position of free swimming corpuscles from one picture to the next. In the period during which the corpuscle remains hanging on the edge the rate of flow does not exceed 0.12 mm. per second (400 mm. per hour), a progress which is too slow to be followed by the naked eye. The pressure brought about by a velocity of this order must be extremely slight.

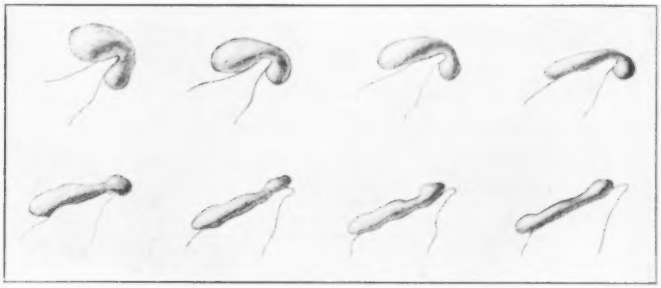
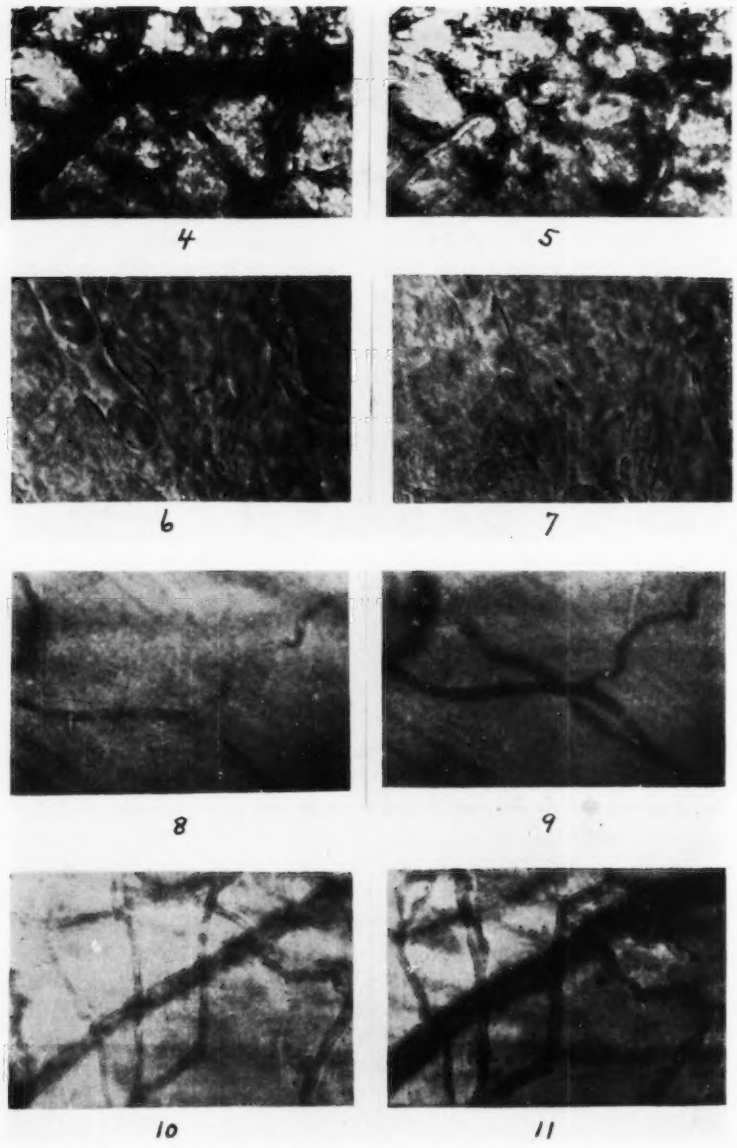


Fig. 3

To show the contraction of an artery the web of the frog has been selected although the vessels of this tissue are somewhat obscured by the numerous black pigment cells. By means of the device shown in figure 2 a drop of adrenaline 1–1000 is placed on the skin over one of the comparatively large arteries issuing directly from the toe. The drop of fluid causes the picture to get out of focus for a brief period. It is restored when the drop diffuses in, and shortly afterwards the artery begins to contract. Figure 5 shows that only the proximal part of the artery contracts strongly while the distal end is scarcely affected. This is a case of the normal insensibility to adrenaline exhibited by the smaller arterial branches in the skin of the frog (3).

Contractions of capillaries in the web have been obtained by faradic stimulation of the sympathetic chain (4) and photographed by the 8 mm. apochromate (90 diameters). In a series of experiments the excised nictitating membrane of a frog has been placed in saline on tinfoil electrodes and stimulated by weak faradic currents as described by Steinach and Kahn (5). These experiments have been photographed by a water-immersion lens at a magnification of 175. There is of course no blood flow in the vessels before the current is put on, but the stimulus brings about contraction, first of the



Figs. 4 and 5. Artery in the web of Rana temporaria before and after the application of a drop of adrenaline ($\frac{1}{1}$).

Figs. 6 and 7. Capillaries in nictitating membrane before and after electrical stimulation ($\frac{1}{1}$).

Figs. 8 and 9. Capillaries in tongue of Rana esculenta before and after mechanical stimulation ($\frac{1}{1}$).

Figs. 10 and 11. Capillaries in frog's tongue before and after application of urethane ($\frac{1}{1}$).

arteries and a little later of the capillaries, inducing a flow which is rapid at first, but soon stops when the arterial contraction is complete. In one of the pictures (figs. 6 and 7) a small gland is also seen to contract.

Opening up and dilatation of capillaries and larger vessels after mechanical stimulation (6) is illustrated in two pictures from the frog's tongue. In one photographed at a low magnification (15 diameters) the surface of the tongue is scratched manually with a needle, and after the stimulus a large number of vessels gradually make their appearance. In the other (40 diameters) the device, figure 2, is used to prick the capillary, visible in the field, thrice in rapid succession causing, after the usual period of latency, a dilatation of this vessel and the opening and gradual dilatation of a second capillary in a slightly deeper position (figs. 8 and 9).

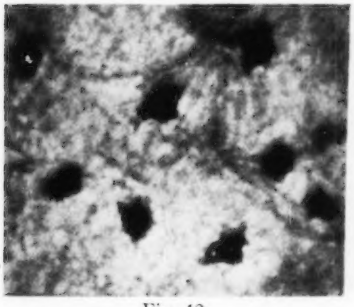


Fig. 12

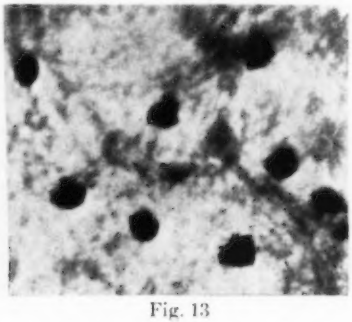


Fig. 13

Figs. 12 and 13. Black pigment cells in web of Rana temporaria before and after stopping of the blood supply for 10 minutes. Contraction caused by lack of pituitary hormone ($\frac{270}{1}$).

In the following experiments a small drop of 15 per cent urethane has been placed on the mucous membrane of the tongue to produce dilatation of the capillaries (6). These experiments are difficult to photograph, because the field will very often get out of focus by an action of the urethane on the muscles of the tongue. In successful experiments the capillaries are dilated to the point of becoming permeable to blood plasma which will filter out and leave a mass of corpuscles, producing a typical stasis (figs. 10 and 11).

In the last experiment the web again is photographed and a pneumatic arrangement is provided, by which the femoral artery can be compressed in the thigh so as to bring the circulation in the web to a standstill. Before the compression there is a rapid flow in narrow capillaries. The compression is maintained for 10 minutes and during the greater part of this period the film is of course stopped. The absence of blood flow brings about a deficiency in pituitary hormone causing some contraction of the

melanophores of the web (figs. 12 and 13) and some relaxation of the capillary walls (7). When the compression is removed the blood flow starts again and the relaxed capillaries become visibly dilated.

SUMMARY

Methods are described for obtaining microkinematographic records of the circulation in transparent tissues.

A kinema film illustrating the main facts of capillary circulation and capillary reactions is described.

BIBLIOGRAPHY

(1) NEUHAUSS: Photogr. Rundschau und Mitt., 1913, l, 374.
(2) KROGH: The anatomy and physiology of capillaries. New Haven, 1922, 59.
(3) KROGH: Journ. Physiol., 1921, lv, 419.
(4) KROGH, HARROP AND REHBERG: Journ. Physiol., 1922, lvi, 181.
(5) STEINACH AND KAHN: Pflüger's Arch., 1903, xcvii, 105.
(6) KROGH: Journ. Physiol., 1920, liii. 399.
(7) KROGH: Anatomy and physiology of capillaries, 141–167.

AN INSULIN-LIKE MATERIAL IN VARIOUS TISSUES OF THE NORMAL AND DIABETIC ANIMAL[1]

C. H. BEST, R. G. SMITH AND D. A. SCOTT

From the Insulin Division of the Connaught Laboratories, University of Toronto

Received for publication December 15, 1923

Immediately after insulin had been obtained from the degenerated pancreas of the dog (1), other tissues were treated by the same procedure as that used in the preparation of the pancreatic extract. The administration to diabetic dogs of extracts of liver and spleen produced negative results. The results obtained from the injection of thymus and thyroid extracts were inconclusive.

We propose in this communication to report the effect upon the blood sugar level of normal rabbits produced by the subcutaneous administration of extracts of thymus, submaxillary gland, thyroid, spleen, liver, brain and muscle tissue from the ox, and of extracts of liver, heart muscle, skeletal muscle, blood and some other tissues from the normal and diabetic dog. In several instances an extract from one of these sources was administered to a diabetic dog. Our results demonstrate the presence of insulin in various tissues other than the pancreas.

METHODS. Various methods have been used in the preparation of these extracts. All of these have been described in a recent article from this laboratory (2). The benzoic acid method (3) was used in the preparation of the majority of the extracts of ox tissue and in all of those of dog tissue. The procedure was as follows: The tissues were removed from the animal as soon as possible after its death and were immediately minced. To every 100 grams of minced material 360 cc. of 95 per cent alcohol, 40 cc. of water and 1.3 cc. of concentrated H_2SO_4 were added. The mixture was stirred occasionally during a period of four hours and was then filtered. The filtrate was concentrated in vacuo to about one-fifth of the original volume. To the concentrate one-half volume of 25 per cent sodium benzoate was added. Concentrated hydrochloric acid (about $\frac{1}{30}$ volume) was then added and the precipitate of benzoid acid which formed was collected in a Buchner funnel. A second precipitate was secured by the addition of more hydrochloric acid to the filtrate. Three precipitations were carried out. The precipitates were mixed and washed two or three times with a

[1] A preliminary communication on this subject appeared in the Journal of the American Medical Association, August 4, 1923.

saturated aqueous solution of benzoic acid. The washed precipitates were then treated with 80 per cent alcohol and the mixture filtered. The filtrate was evaporated nearly to dryness in vacuo. The residue was treated with a volume of sulphuric ether sufficient to dissolve the benzoic acid. The solution was transferred to a separatory funnel and the aqueous layer after separation from the supernatant ether was washed twice with ether. Traces of ether were removed from the aqueous layer by vacuum distillation and the watery solution was concentrated to a convenient volume.

The process for blood was the same as for the solid tissues with the exception that four and one-half volumes of 95 per cent alcohol were added to each volume of blood instead of the quantity above stated. One cubic centimeter of concentrated H_2SO_4 was added for every 100 cc. of blood. Subsequent details were the same as described above for the other tissues.

The thymus glands from the ox and, in some experiments, other tissues from this source were processed by the method which was originally used to prepare insulin in a form suitable for repeated administration to patients (4). Briefly, this method consists in extracting the glands with acid alcohol; removing the alcohol and some of the water in vacuo; making the concentrate up to 80 per cent with alcohol; and precipitating the insulin with associated material from alcoholic solution by raising the concentration of alcohol to approximately 92 per cent. The precipitate is dissolved in water and administered (either with or without further purification) to rabbits or dogs.

The Shaffer-Hartmann (5) method was used in the determination of the amounts of sugar in the blood samples. The urinary sugar was estimated by Benedict's method. The Kjeldahl method was used in estimating total nitrogen of the urine. Leathes' modification of the Kumagawa-Sato method and a modified Pflüger method were used in estimating the fat and glycogen content of the liver.

The unitage per 100 grams of tissues was calculated by means of the formula now used by the testing department of the Insulin Committee, University of Toronto. This formula, which has been used by the Committee under the direction of Prof. J. J. R. Macleod, is designed to give results in terms of a unit, the definition of which is—one-third the amount of material required to lower the blood sugar of a rabbit weighing 2,000 grams which has been starved twenty-four hours, from the normal level to 0.045 per cent over a period of five hours. In those experiments where the rabbit took convulsions, but recovered from them spontaneously during the test, blood sugar estimations at the usual intervals were made. When the animal exhibited severe convulsions which persisted throughout the entire experiment we assumed that the blood sugar level at the remaining determinations would be approximately the same as at the estimation which was made after the onset of the convulsions. Obviously we have only obtained

an approximate idea of the unitage in these cases. We believe, however, that the suggested figure is not too high an estimate. In many experiments, as will be observed, we had only small quantities of insulin-containing extract to work with.

EXPERIMENTAL RESULTS. In table 1 we have collected the results of a number of experiments some of which were carried out nearly a year ago. As the blood sugars in these preliminary experiments were not estimated at intervals corresponding to those now used by the testing department of the Insulin Committee, we will not attempt to calculate the yield of insulin-

TABLE 1

TISSUE	WEIGHT OF RABBIT	NORMAL BLOOD SUGAR	LOWEST BLOOD SUGAR OBSERVED	CONVULSIONS
	grams	*per cent*	*per cent*	
Thymus	1850	0.104	0.035	Very severe
Thymus	2000	0.124	0.048	Severe
Thymus	1850	0.104	0.036	Severe
Thymus	2000	0.110	0.035	Mild
Thymus	1500	0.110	0.054	None
Thymus	1400	0.098	0.066	None
Thymus	1750	0.114	0.060	None
Thymus	1800	0.136	0.079	None
Thymus	1700	0.101	0.066	None
Thymus	1850	0.104	0.054	None
Submaxillary	1850	0.118	0.060	Mild
Submaxillary	1850	0.118	0.066	Severe
Thyroid	1650	0.130	0.072	None
Spleen	1450	0.136	0.060	None
Liver	1000	0.142	0.079	None
Liver	1450	0.104	0.066	None
Liver	1700	0.104	0.060	None
Brain	2000	0.092	0.072	None
Brain	1700	0.110	0.066	None
Muscle	1900	0.124	0.066	None
Muscle	1800	0.092	0.079	None

like material in terms of units per 100 grams of tissue. We have recorded, however, the normal blood sugar and the lowest percentage of blood sugar observed during each experiment. This latter value was always observed in less than five hours after the material being tested had been injected.

More recent experiments with extracts of calves' thymus glands show that from twenty-five to fifty units of insulin per kilogram of tissue may be extracted from this material by the routine procedure which we use in preparing insulin from beef or pork pancreas.

In the following protocols we have attempted to report the immediate history of the experimental animal and have recorded in tabular form the

results obtained by administering extracts of certain of its tissues to normal rabbits or diabetic dogs.

Dog 4. (White.) Weight was 9 kilos. Complete pancreatectomy was performed under ether anesthesia on July 14. July 17, blood sugar 3:30 p.m. was 0.250 per cent, urinary volume was 490 cc., sugar excretion was 9.24 grams. July 18, blood sugar was 0.224 per cent, volume of urine was 405 cc., and sugar excretion was 17.2 grams. July 19, blood sugar was 0.236 per cent, volume of urine 246 cc., and sugar excretion 14.6 grams. July 20, blood sugar was 0.236 per cent, volume of urine 200 cc., and sugar excretion 1.11 grams. This animal was fed approximately $\frac{3}{4}$ pound of lean beef per day. On some days however the meat was not eaten. The blood sugar on July 20 was estimated, as on the other days, at 3:30 p.m. The value was, as stated above, 0.236 per cent. At 4 p.m. 7 cc. of an extract of liver were administered subcutaneously. At 7 p.m. blood sugar was 0.212 per cent; 9 p.m., blood sugar 0.174 per cent; 11 p.m., blood sugar 0.167 per cent; 1 a.m., blood sugar 0.167 per cent. July 21, the volume of urine was 100 cc. and the sugar excreted was 1.090 grams. July 22, blood sugar was 0.274 per cent; volume of urine 65 cc., and sugar excreted 21.4 grams. July 23, 10 a.m., volume of urine was 180 cc. and the sugar excreted 0.80 gram. These figures apply to the period between 3:30 p.m. July 22 and 10 a.m. July 23. Blood sugar July 23, 10 a.m., 0.219 per cent. Ten cubic centimeters of the same preparation of liver as above were administered subcutaneously Blood sugar at 12 m. was 0.219 per cent; 2 p.m., 0.160 per cent; 4 p.m., 0.154 per cent; 6 p.m., 0.224 per cent; 8 p.m., 0.280 per cent; 10 p.m., 0.256 per cent; July 24, 11 a.m., blood sugar was 0.240 per cent, sugar excreted during previous 24 hours, 2.27 grams in a volume of 40 cc. of urine. On July 25 at 10 a.m. the animal was killed. Blood sugar of heart blood was 0.174 per cent. The operation wound of this animal became badly infected toward the end of the experiment and a satisfactory autopsy was not obtained. The consistently high blood sugar of the animal argues in favor of the completeness of the pancreatectomy. The diminished volume of urine and amount of sugar excreted on certain days suggest renal complications. We believe, but cannot be absolutely certain, that the pancreas was completely extirpated. The values obtained by extracting the tissues for insulin are included in table 2.

Dog 8. Black pup. Weight was 8 kilos. Complete pancreatectomy was performed on July 23. July 25 blood sugar was 0.205 per cent. July 26, blood sugar was 0.243 per cent; and sugar excreted 26.3 grams on a diet of $\frac{1}{2}$ pound of lean meat per day. Urine volume was 750 cc. July 27, blood sugar was 0.224 per cent and sugar excreted was 15.9 grams in a volume of 590 cc. of urine. The animal was on a starvation diet. Dog killed July 27. Autopsy showed no pancreas in the gross. Tissues were treated to extract insulin.

Dog 9. Yellow collie. Weight was 20 kilos. Complete pancreatectomy was performed on August 2. Blood sugar August 3 was 0.243 per cent. On August 4, 5, 6 and 7, the blood sugar, sugar excretions and urinary volumes were as follows: 0.180 per cent, 1850 cc., 35.6 grams; 0.205 per cent, 715 cc., 32.5 grams; 0.212 per cent, 625 cc., 40.1 grams; 0.274 per cent, 1250 cc., 32.1 grams. The animal was on a lean meat diet of approximately $\frac{1}{2}$ pound per day. The animal was killed on August 7. An autopsy showed no trace of pancreas in the gross. The tissues of the animal were treated to extract insulin.

Dog 10. Female Airedale. Weight 14.5 kilos. Complete pancreatectomy was performed on August 2, 3 p.m. August 4, 11 a.m., blood sugar was 0.199 per cent. Sugar excreted during previous twenty-four hours was 35.7 grams in 600 cc. of urine. August 5, blood sugar was 0.212 per cent, 730 urine, 34.8 grams sugar. August 6,

TABLE 2

Dog 4

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*					
72 grams liver	7	2600	0.136	0.085	0.085	0.092	2.91
72 grams liver	7	1500	0.118	0.060	0.054	0.072	2.39
72 grams muscle	5	2500	0.104	0.054	0.054	0.054	4.41
72 grams muscle	5	2000	0.110	0.083	0.066	0.085	1.98
72 grams heart	13	2200	0.124	0.092	0.060	0.066	3.17
100 cc. blood	8	2000	0.079	0.054	0.048	0.040*	2.82
100 cc. blood	8	2000	0.142	0.079	0.066	0.054	2.35

* The asterisk indicated that the animal took convulsions. The unitage in these experiments is therefore only approximate.

TABLE 3

Dog 8

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*					
48 grams liver	2.6	1700	0.118	0.104	0.098	0.130	0.80
100 grams liver	5.2	1600	0.105	0.092	0.085	0.060	1.04
48 grams muscle	4.1	2200	0.130	0.048	0.060	0.040*	6.56
48 grams muscle	4.1	1800	0.110	0.040	0.040	0.054*	5.62
48 grams heart	4.1	2100	0.130	0.078	0.078	0.066	1.70
60 cc. blood	6.3	2000	0.130	0.110	0.110	0.110	1.18
120 cc. blood	12.6	2100	0.130	0.079	0.079	0.066	1.70

TABLE 4

Dog 9

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*					
80 grams liver	6.0	1550	0.098	0.072	0.066	0.048	1.50
100 grams liver	7.5	1750	0.104	0.066	0.072	0.085	1.33
60 grams muscle	5.4	1600	0.104	0.110	0.079	0.110	0.54
95 grams muscle	8.5	1750	0.098	0.104	0.079	0.104	0.10
50 grams heart	5.0	1500	0.085	0.124	0.085	0.092	
70 grams heart	7.0	1500	0.098	0.092	0.110	0.104	
60 cc. blood	4.2	1800	0.104	0.079	0.092	0.085	1.45
100 cc. blood	7.0	1700	0.130	0.079	0.079	0.104	1.69

blood sugar was 0.212 per cent, 1100 cc. urine, 83.3 grams sugar. August 7, blood sugar 0.230 per cent, 500 cc. urine, 26.8 grams sugar. Diet was approximately ½ pound of lean meat per day. Animal killed on August 7. No pancreas was present at autopsy. Tissues were treated to extract insulin.

Dog 15. Black and white. Weight was 14.5 kilos. Complete pancreatectomy was performed on August 27. As this animal was observed over a somewhat longer

TABLE 5

Dog 10

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*					
100 grams liver.....	9.0	1750	0.098	0.092	0.098	0.085	0.30
100 grams liver.....	9.0	2000	0.118	0.104	0.085	0.083	1.11
50 grams muscle	7.0	1500	0.124	0.079	0.092	0.085	2.21
80 grams heart.....	10.0	1800	0.092	0.054	0.072	0.048	2.44
125 cc. blood.....	12.5	2400	0.118	0.066	0.072	0.054	2.13
125 cc. blood.....	12.5	2000	0.118	0.072	0.060	0.054	1.84

TABLE 6

Dog 15

DATE	TIME	BLOOD SUGAR	VOLUME OF URINE	AMOUNT OF SUGAR	NITROGEN	G/N	DIET
		per cent	*cc.*	*grams*	*grams*		
August 28	3 p.m.	0.186	800	2.1			½ lb. meat
August 29	3 p.m.	0.212	1250	48.9			½ lb. meat
August 30	3 p.m.	0.242	1050	42.7			½ lb. meat
August 31	3 p.m.	0.180	660	9.1			Starved
September 1	3 p.m.		1700	54.6			1 lb. meat
September 2	3 p.m.	0.224	1750	39.5			1 lb. meat
September 3	3 p.m.	0.186	900	8.1			Starved
September 4	3 p.m.	0.200	760	9.3	3.9	2.38	Starved
September 5	2 p.m.	0.224	850	3.2	4.2	1.31	Starved
September 6	3 p.m.	0.230	490	2.9	2.5	1.16	Starved
September 7	3 p.m.	0.242	1160	1.7	4.5	0.38	Starved
September 8	3 p.m.	0.230	350				Starved
September 9	3 p.m.	0.266	565	4.3	3.5	1.23	Starved
September 10	3 p.m.	0.254	500	5.0	2.15	2.32	Starved

period than others, the observations on blood sugar, sugar excretion and nitrogen excretion will be reported in tabular form.

The animal was killed on September 10. Autopsy showed no pancreas in the gross. Serial sections were made of the duodenum. Mr. Bowie, B.Sc. (Pathology), of the Department of Physiology, reported that no pancreatic tissue, with the exception of a small nodule less than 1 mm. square, could be found. The tissues of the animal were treated to extract insulin. The pancreas was extracted immediately after the pancreatectomy, August 27.

Dog. 11. Short hair. Normal dog, weighing 27 kilos, was killed on August 2. Tissues were treated to extract insulin.

Dog 12. Normal dog, weighing 7.8 kilos, was killed August 2. Tissues were treated to extract insulin.

TABLE 7

Dog 15

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*	*per cent*	*per cent*	*per cent*	*per cent*	
7 grams pancreas...	8.5	1500	0.104	0.060	0.060	0.048	17.4
10 grams pancreas...	12.0	1750	0.085	0.035	*	*	18.8(?)
80 cc. blood.....	6.0	1250	0.098	0.066	0.072	0.060	0.94
80 cc. blood.....	6.0	1650	0.110	0.079	0.060	0.072	1.27
100 cc. blood.....	7.0	1700	0.092	0.040	0.066	0.066	1.27
100 cc. blood.....	7.0	1700	0.118	0.040	0.060	0.092	1.26
100 grams liver.....	10.0	2000	0.079	0.048	0.060	0.054	1.47
100 grams liver.....	10.0	1700	0.098	0.035	*	*	2.12(?)
62 grams liver.....	7.0	1150	0.092	0.060	0.048	0.048	1.61
72 grams heart.....	7.0	2250	0.098	0.066	0.066	0.072	1.77
100 grams muscle.....	9.0	1450	0.110	0.066	0.054	0.048	1.20
65 grams kidney.....	19.0	2150	0.103	0.095	0.072	0.040	1.94
65 grams lung.....	11.0	2000	0.098	0.037	0.048	*	3.19(?)
10 grams spleen.....	12.0	1350	0.092	0.072	0.072	0.072	3.73

TABLE 8

Dog 11

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*	*per cent*	*per cent*	*per cent*	*per cent*	
50 grams liver.....	8.8	1800	0.104	0.054	0.079	0.085	2.84
90 grams liver.....	15.8	1800	0.110	0.066	0.048	0.048	2.58
50 grams muscle.....	9.5	1800	0.118	0.092	0.098	0.110	1.33
42 grams muscle.....	8.0	1600	0.098	0.079	0.079	0.079	2.05
50 grams heart.....	20.0	1800	0.130	0.060	0.045	0.048*	5.02
15 grams pancreas....	12.0	1700	0.118	0.048	0.048	0.048*	16.00
40 cc. blood.....	5.5	1600	0.124	0.124	0.072	0.124	2.96
60 cc. blood.....	8.0	1600	0.124	0.054	0.040	0.066	3.59
70 cc. blood.....	9.5	2000	0.130	0.066	0.040	0.045	4.00

Dog 13. Large, yellow, normal dog, weighing 25.9 kilos, was killed August 22. Tissues were extracted with alcohol and the extract purified by the benzoic acid process.

Dog 14. Black pup. Normal dog, weighing 12 kilos, was killed August 22. The tissues were treated in exactly the same manner as in the other experiments of this series.

Dog 16. Airedale pup. Normal dog, ill-nourished and irritable, weighing 9 kilos, was killed September 5. Tissues were treated to extract insulin.

Dog 17. Normal Airedale, weighing 17 kilos, was retained in the third stage of ether anesthesia for 30 minutes and then killed by excess ether. Definite amounts of tissues were removed and treated to extract insulin.

TABLE 9

Dog 12

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*	*per cent*	*per cent*	*per cent*	*per cent*	
50 grams liver.....	5.0	1850	0.130	0.066	0.054	0.079	4.32
100 grams liver.....	10.0	1900	0.110	0.066	0.054	0.066*	2.19
46 grams muscle.....	5.5	1700	0.118	0.085	0.048	0.054	4.22
100 grams muscle.....	12.3	1800	0.118	0.035	0.048	0.035*	2.70
55 grams heart.....	15.0	1800	0.104	0.048	0.048	0.040	4.91
10 grams pancreas...	7.5	1800	0.098	0.040	0.048	0.079	23.43
10 grams pancreas...	7.5	1750	0.104	0.054	0.018	0.018	26.25
60 cc. blood.....	6.0	1900	0.098	0.040	0.035	0.072	4.38
60 cc. blood.....	6.0	1800	0.098	0.072	0.048	0.048	3.57
65 cc. blood.....	6.5	1850	0.092	0.048	0.048	0.072	3.27

TABLE 10

Dog 13

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*	*per cent*	*per cent*	*per cent*	*per cent*	
10 grams pancreas...	7.5	1600	0.130	0.035	0.040*		26.0+(?)
8 grams pancreas...	6.0	1600	0.124	0.000*			26.0++(?)
8 grams pancreas...	6.0	1650	0.124	0.040*			26.0++(?)
100 grams liver.....	18.0	1650	0.110	0.035	0.054*		1.70
100 grams liver.....	18.0	1650	0.118	0.035	0.048	0.035	1.73
50 grams liver.....	9.0	1550	0.110	0.072	0.048	0.060	2.38
62 grams heart.....	11.0	1500	0.104	0.048	0.060	0.060	2.03
62 grams heart.....	11.0	1650	0.110	0.079	0.072	0.072	1.52
60 cc. blood.....	15.0	1650	0.104	0.048	0.040*		2.84+(?)
60 cc. blood.....	15.0	1700	0.110	0.048	0.035*		3.09+(?)
60 cc. blood.....	15.0	1600	0.110	0.054	0.040		2.66+(?)

Dog 21. Normal black and white terrier, was retained in the third stage of ether anesthesia for 1 hour and 30 minutes and then killed by excess ether. Definite amounts of tissues and blood were removed and treated to extract insulin.

Dog 22. Normal, female Airedale was retained in the third stage of ether anesthesia for 45 minutes and then killed by excess ether. Definite amounts of tissues and blood were removed and extracted for insulin.

Dog 20. Black and white adult dog, weighing 8.6 kilos, was completely depancreatized on September 20, at 8 p.m. The effects upon blood and urinary sugar, G/N ratio, and the general condition of the animal produced by the subcutaneous administration of extract of thymus are shown in tabular form.

Animal was killed 4 p.m., October 3.

TABLE 11

Dog 14

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*	*per cent*	*per cent*	*per cent*	*per cent*	
80 grams muscle.....	16.0	1650	0.104	0.095	0.082	0.083	0.60
60 grams muscle.....	12.0	1650	0.110	0.104	0.101	0.118	0.21
100 grams muscle.....	20.0	1700	0.104	0.095	0.088	?	0.49(?)
80 grams liver.....	11.0	1750	0.104	0.040	0.044	0.104	1.52
80 grams liver.....	11.0	1700	0.118	0.035	0.044*		2.27(?)
80 grams liver.....	11.0	1700	0.168	0.040	0.038	0.033*	2.25
60 cc. blood.....	14.0	1750	0.104	0.040*			2.70(?)
60 cc. blood.....	14.0	1650	0.098	0.018*			3.37(?)
40 cc. blood.....	9.3	1500	0.118	0.044*			3.75(?)
40 cc. blood.....	9.3	1600	0.110	0.063	0.063	0.072	2.71
80 grams heart.....	22.0	1800	0.110	0.088	0.079	0.104	0.70
10 grams pancreas...	12.5	1850	0.140	0.060	0.048	0.072	15.58
7 grams pancreas...	8.8	1850	0.104	0.066	0.060	0.066	17.92

TABLE 12

Dog 16

TISSUE	VOLUME OF INJECTION	WEIGHT OF RABBIT	N.B.S.	B.S. 1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	cc.	*grams*	*per cent*	*per cent*	*per cent*	*per cent*	
5 grams pancreas...	7.5	1500	0.124		0.048	*	
10 grams pancreas...	14.0	2400	0.118	0.060	0.035	*	23.3(?)
60 cc. blood.....	8.5	2250	0.118	0.098	0.110	0.110	0.62
120 cc. blood.....	17.0	1800	0.110	0.085	0.092	0.088	0.51
75 grams heart.....	13.0	2300	0.148	0.073	0.079	0.098	2.02
70 grams liver.....	7.0	2350	0.110	0.110	0.085	0.104	1.08
70 grams liver.....	7.0	2350	0.104	0.092	0.110	0.110	
100 grams muscle.....	9.5	1800	0.092	0.048	0.066	0.044	1.49
100 grams muscle.....	9.5	1850	0.118	0.057	0.072	0.069	1.32

The liver of dog 20 was analyzed for glycogen and fat. The liver contained 6 per cent glycogen and 8.45 per cent fatty acids.

Dog 25. Large Airedale. Weight 23.5 kilos. Pancreatectomy performed October 31, 5 p.m. Weight of pancreas removed, 35 grams. Dog was starved throughout the experiment.

Dog 26. Demonstrating the action of an extract of diabetic tissues. White bull terrier. Weight 8.6 kilos. Pancreatectomy November 8, 5 p.m. Dog was starved

throughout the experiment. Extracts used for injection were from the tissues and blood of dog 25.

2 cc. of solid tissue extract represents 100 grams of the original tissue.

7 cc. of blood extract represents 100 cc. of the original blood.

TABLE 13

Dog 17

TISSUES	WEIGHT OF RABBIT	N.	1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	grams	*per cent*	*per cent*	*per cent*	*per cent*	
10 grams pancreas.....	1650	0.136	0.098	0.085	0.072	8.77
7 grams pancreas.....	2000	0.142	0.066	0.048	0.092	21.5
5 grams pancreas.....	1750	0.104	0.018	0.040	0.092	32.0
7 grams pancreas.....	1800	0.098	0.066	0.054	0.110	10.2
5 grams pancreas.....	1800	0.098	0.079	0.085	0.066	14.3
100 grams liver.....	1450	0.136	0.118	0.098	0.098	0.48
80 grams liver.....	1700	0.104	0.110	0.098	0.079	0.29
90 grams liver.....	2150	0.110	0.142	0.104		
100 grams muscle.....	1600	0.092	0.110	0.680	0.098	0.10
100 grams muscle.....	1600	0.154	0.085	0.110	0.092	0.85
60 grams muscle.....	2250	0.110	0.066	0.072	0.035(?)	
120 grams heart.....	2200	0.124	0.092	0.092	0.092	0.73
60 cc. blood.....	1700	0.092	0.098	0.085	0.085	0.18
60 cc. blood.....	1700		0.098	0.110	0.092	
120 cc. blood.....	1900	0.104	0.118	0.092	0.098	
145 cc. blood.....	1750	0.118	0.092	0.092	0.072	0.54

TABLE 14

Dog 21

TISSUE	WEIGHT OF RABBIT	N.	1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	grams	*per cent*	*per cent*	*per cent*	*per cent*	
110 cc. blood.....	2000	0.124	0.085	0.085	0.110	0.71
110 cc. blood.....	1600	0.133	0.066	0.063	0.083	1.02
110 cc. blood.....	1650	0.104	0.072	0.098	0.098	0.20
80 grams heart.....	1850	0.095	0.079	0.085	0.104	0.42
100 grams liver.....	1950	0.118	0.066	0.066	0.104	1.69
100 grams liver.....	1975	0.114	0.092	0.110	0.130	0.32
100 grams muscle.....	1600	0.118	0.054	0.079	0.084	1.08
185 grams muscle.....	1700	0.110	0.072	0.072	0.085	0.49
10 grams pancreas.....	1700	0.110	0.040*			17.0?
6 grams pancreas.....	1700	0.118	0.025*			32.0?

From our recent experience with depancreatized dogs we are convinced that certain of these animals will live for a much longer period than is generally supposed.

Dog 19, a collie pup weighing 9 kilos, was depancreatized on September 19. The weight of pancreas removed was 16 grams.

Dog killed November 13. Tissues and blood were extracted for insulin. On injection into rabbits the tissue extract showed positive results. The most marked effect was obtained from blood. An extract of 200 cc. of blood injected into 1800 grams rabbit gave the following result: Normal blood sugar, 0.132 per cent; blood sugar 1½ hours after injection, 0.057 per cent; 3 hours after injection, 0.018 per cent, and 5 hours after injection, 0.035 per cent.

The autopsy on this animal was conducted by the Pathological Department of the Toronto General Hospital. We include their report:

"Report of Autopsy done on Dog 19, November 13, 1923.

"This post-mortem was done with the object of determining the presence or absence of any pancreatic tissue following a total pancreatectomy done some eight weeks prior to the death of the animal.

"*Gross.* A number of small nodules could be felt in the mesentery and about the region formerly occupied by the pancreas.

TABLE 15

Dog 22

TISSUE	WEIGHT OF RABBIT	N.	1½ HOURS	3 HOURS	5 HOURS	UNITS PER 100 GRAMS OF TISSUE
	grams	*per cent*	*per cent*	*per cent*	*per cent*	
100 cc. blood	1800	0.092	0.107	0.085	0.101	
100 cc. blood	1700	0.135	0.108	0.110	0.121	
9 grams pancreas	1800	0.104	0.069			9.6
9 grams pancreas	1600	0.104	0.051	0.072	0.092	9.6
65 grams heart	1600	0.118	0.072	0.088	0.092	0.6
100 grams muscle	2400	0.118	0.085	0.066	0.079	1.35
100 grams muscle	2200	0.118	0.098	0.079	0.110	0.66
100 grams muscle	1500	0.114	0.124	0.114	0.124	
100 grams liver	1850	0.111	0.114	0.104	0.088	0.24
100 grams liver	1800	0.103	0.095	0.101	0.095	0.19
100 grams liver	1650	0.110	0.066	0.079	0.104	0.10
100 grams liver	1600	0.121	0.130	0.098	0.110	

"There were a couple of small thickened areas in the wall of the duodenum. Otherwise nothing was found on gross examination that indicated the presence of pancreatic tissue.

"*Microscopic.* Section of the nodules in the mesentery show them to be lymph glands.

"One of the nodules in the wall of the duodenum is seen to be a small portion of pancreatic tissue lying partly in the submucosa and extending into the muscular coat, apparently displacing its fibers. This nodule is spherical and 3 mm. in diameter. There are no islets present in the section observed while the characteristic pancreatic tissue was well demonstrated.

(Signed) W. L. Robinson."

We find that a piece of normal pancreas of the same dimensions as the piece found in the submucosa of the duodenum of this animal weighs approximately 0.02 gram. Therefore only one eight-hundredth part of its normal pancreas was left in the animal. Obviously this could have exerted little if any influence upon the insulin content of the tissues.

TABLE 16

Dog 20

DATE	TIME	BLOOD SUGAR	URINE VOLUME	SUGAR EXCRETION	TOTAL N OF URINE	G/N RATIO	INJECTIONS	DIET	REMARKS
		per cent	*cc.*	*grams*	*grams*				
Sept. 25	9:00 a.m.	0.205	450	19.9	6.7	3.0		Starved	Animal very weak
	11:30 a.m.						10 cc. urine extract		
	2:00 p.m.	0.197							Animal suffering from severe infection of both eyes (conjunctivitis)
	4:30 p.m.	0.192							
	8:00 p.m.	0.186							
Sept. 26	9:00 a.m.		285	18.6	6.3	3.0		Starved	
Sept. 27	9:00 a.m.	0.253	510	24.3	9.2	2.6		Starved	Animal could not stand without support
	10:30 a.m.						4 cc. thymus extract		
	3:30 p.m.	0.256							
	4:30 p.m.						10 cc. thymus extract		
	9:00 p.m.	0.186							
Sept. 28	9:00 a.m.	0.253	185	5.7	2.5	2.3			Condition of animal improving
	9:30 a.m.						10 cc. thymus extract		
	12:00 a.m.	0.121							
	2:00 p.m.						10 cc. thymus extract		
	2:45 p.m.	0.079							
	4:30 p.m.	0.048							
	8:30 p.m.	0.060							

Sept. 29	9:00 a.m.	0.286	545	Neg.	4.8		10 cc. thymus extract	½ lb. meat	
Sept. 30	10:30 a.m.						8 cc. thymus extract	¼ lb. meat	Animal very lively Eyes much improved
Oct. 1	9:00 a.m.	0.110					10 cc. thymus extract	½ lb. meat	
Oct. 2	9:00 a.m.	0.212	365	5.6			9 cc. thymus extract	40 grams glucose (stomach tube)	
	10:30 a.m.							½ lb. meat	
	5:00 p.m.	0.258					8 cc. thymus extract	50 grams glucose (stomach tube)	Animal appears strong and eats well
	11:30 p.m.						7 cc. thymus extract	40 grams glucose (stomach tube)	
Oct. 3	2:00 p.m.	0.082					15 cc. thymus extract	¼ lb. meat. 40 grams glucose	

TABLE 17

Dog 25

DATE	TIME	BLOOD SUGAR	URINE VOLUME	SUGAR EXCRETION	TOTAL N	$\frac{G}{N}$ RATIO	INJECTIONS	REMARKS
		per cent	*cc.*	*grams*	*grams*			
Nov. 3	9:00 a.m.	0.224	785	56.7	16.9	3.35		Urine for 12 hour period
	9:30 a.m.						25 cc. urine extract #1 subc.	
	12:30 p.m.	0.186						
	2:30 p.m.	0.192						
	4:30 p.m.	0.180						
	7:30 p.m.	0.148						
Nov. 4	9:00 a.m.		730	20.9	14.1	1.48		Urine for previous 24 hours
Nov. 5	9:00 a.m.		1000	79.3	20.5	3.87		Urine for previous 24 hours
Nov. 6			1030	80.4	21.75	3.69		Urine for previous 24 hours
Nov. 6	4:30 p.m.	0.212	450	37.5	11.05	3.39	25 cc. urine extract #2 subc.	Urine 9 a.m.–4:30 p.m.
	7:30 p.m.	0.205						
	11:30 p.m.	0.268					25 cc. urine extract #1 subc.	
Nov. 7	4:30 p.m.	0.142	765	55.9	15.05	3.71		Urine for previous 24 hours
	10:30 p.m.	0.262						
Nov. 8	9:00 a.m.		450	33.6	12.96	2.59		Urine 4:30 p.m.–9 a.m.
	12:00 p.m.	0.345	580	50.0	13.38	3.74		Urine 9 a.m.–12 p.m. Dog killed at 12 p.m. Blood and tissues extracted for insulin by method previously described. Blood—800 cc. Muscle—2010 grams. Other tissues—1000 grams

TABLE 18

Dog 26

TABLE 18

Dog. 26

DATE	TIME	BLOOD SUGAR	URINE VOLUME	SUGAR EXCRETION	TOTAL N	$\frac{G}{N}$ RATIO	INJECTIONS	REMARKS
		per cent	*cc.*	*grams*	*grams*			
Nov. 13	9:00 a.m.		630	26.7	7.3	3.6		
	5:00 p.m.	0.228						
Nov. 14	9:00 a.m.		540	16.6	8.8	1.8		
Nov. 15	9:00 a.m.		225	5.1	2.9	1.7		
Nov. 16	9:00 a.m.	0.242	480	11.3	5.5	2.05	35 cc. blood extract subcutaneously	
	12:15 p.m.	0.221						
	4:15 p.m.	0.199						
	5:15 p.m.						20 cc. tissue extract subcutaneously	The animal appeared much brighter during the period in which the blood sugar was low, than in the previous or subsequent period
	8:15 p.m.	0.128						
	11:15 p.m.	0.092						
Nov. 17	9:00 a.m.	0.132	630	8.9	6.3	1.4		
	12:00 a.m.						10 cc. tissue extract subcutaneously	
	2:15 p.m.	0.160						
	4:15 p.m.	0.158						
	5:30 p.m.						15 cc. tissue extract subcutaneously	
	7:30 p.m.	0.066						
Nov. 18	11:00 a.m.	0.148	610	5.1	4.83	1.05		
Nov. 19	9:00 a.m.		470	17.1	7.24	2.4		
Nov. 20	9:00 a.m.		340	11.14	3.91	2.9		

TABLE 19

Dog 19

DATE	BLOOD SUGAR	URINE VOLUME	SUGAR EXCRETION	TOTAL N	$\frac{G}{N}$ RATIO	DIET	REMARKS
	per cent	*cc.*	*grams*	*grams*			
Sept. 20	0.130					Starved	Blood samples taken and urine collected at 9 a.m.
Sept. 21	0.130	253	1.2	3.3	0.36	Starved	
Sept. 22	0.098	640	0.9	4.1	0.22	Starved	Urine samples were for previous 24 hours
Sept. 23		560	1.2	8.4	0.17	½ lb. lean beef daily	
Sept. 24		450	1.8	6.7	0.27		
Oct. 1	0.199					½ lb. lean beef daily	Weight of animal September 30, 10 kilos
Oct. 2		560	35	5.6	6.2	½ lb. lean beef and ½ pint milk fed daily till October 8	On October 8 there was marked conjunctivitis and blood in stools
						Starved Oct. 9–13	The above signs had disappeared by October 13
Oct. 12		210		2.8			
Oct. 13	0.160	130		1.9			
						100 gms. beef and ½ pint milk daily Oct. 13–Nov. 13	Weight of animal October 20, 7.3 kilos
Nov. 2		355	13.9				

TABLE 20

NORMAL DOG'S BLOOD		DIABETIC DOG'S BLOOD			ANESTHETIZED DOG'S BLOOD		
Number of dog	Units per 100 cc.	Number of dog	Days after operation	Units per 100 cc.	Number of dog	Minutes under ether	Units per 100 cc.
11	2.96	4	11	2.82	22	45	0
	3.59			2.35			0
	4.00						
12	4.38	8	4	1.18	21	90	0.71
	3.57			1.70			1.02
	3.27						0.20
13	2.84	9	5	1.45	17	30	0.18
	3.09			1.69			0
	2.66						0
							0.54
14	2.70	10	5	2.31			
	3.37			1.84			
	2.71						
	3.75						
16	0.62	15	14	0.94			
	0.51			1.27			
				1.27			
				1.26			
Average.....	2.93			1.66			0.29

TABLE 21

NORMAL DOG'S LIVER		DIABETIC DOG'S LIVER			ANESTHETIZED DOG'S LIVER		
Number of dog	Units per 100 grams	Number of dog	Days after operation	Units per 100 grams	Number of dog	Minutes under ether	Units per 100 grams
11	2.84	4	11	2.91			
	2.58			2.39	22	45	0.24
							0.19
							0.10
							0.00
12	4.32	8	4	0.80	21	90	1.69
	2.19			1.04			0.32
13	1.70	9	5	1.50	17	30	0.48
	1.73			1.33			0.29
	2.38						0.00
14	1.52	10	5	0.30			
	2.27			1.11			
	2.25						
16	1.08	15	14	1.47			
	0.00			2.12			
				1.61			
Average.....	2.08			1.51			0.53

DISCUSSION. The method we have used in preparing the extracts of dog tissue was not adopted because it yielded a greater amount of insulin-like material per gram of tissue than any other method. We decided to utilize this method because we found that the results obtained when it was applied to the routine preparation of insulin from pancreas were comparatively consistent. In the consideration of our results it must be borne in mind that the method employed had been evolved for the extraction and purification of insulin from pancreatic tissue. It is possible, therefore, that by more appropriate procedures much larger yields of insulin-like material might be obtained from tissues other than the pancreas.*

If all our estimations are averaged they indicate that at least 21.5 units of insulin can be extracted from each 100 grams of normal dog's pancreas, by the method we have used. In a like manner normal dog's blood appears to yield 2.93 units and diabetic dog's blood 1.66 units per 100 cc. of tissue. In certain preliminary experiments we were unable to demonstrate insulin-like material in the blood of diabetic dogs who were suffering from complicating infections. Normal liver yields 2.07 units while diabetic liver yields 1.51; normal muscle 1.68, and diabetic muscle 2.83 units per 100 grams. Normal dog's heart muscle assays 2.70 and diabetic heart muscle 1.51 units per 100 grams of tissue. In the three experiments in which we attempted to assay the insulin content of dog's tissues after ether anesthesia the results indicate that certain of these contain less active material than normal or diabetic tissues.

The rabbit test is very unsatisfactory but appears to be the best we have available at the present time. *Since the accuracy of the method of calculating the unitage has not been completely proven, our results should be criticised on the basis of the blood sugar lowering effect of the injected material rather than on that of the calculated unitage.*

We realize that many more experiments in which a more accurate method of testing is employed, are needed before definite conclusions can be drawn concerning the insulin content of the various tissues under different experimental conditions. However, the tests made upon extracts of blood or liver appear to give fairly comparable results for the same preparation of blood or liver and it appears that the amounts of insulin-like material in these tissues of diabetic or aneesthtized animals are definitely smaller than in the same tissues from normal dogs. The results obtained by the administration of extracts of muscle are rather more discordant than those obtained with other tissues. It is interesting, however, that the highest yield of insulin-like material from muscle tissue, both average and individual, was obtained from muscle of the diabetic animal. We do not suggest on the basis of these few experimental results

*It is doubtful whether the rabbit method of testing is sufficiently accurate to necessitate the use of fractions to express the unitage per 100 grams of tissue.

that the insulin content of diabetic muscle may be greater than that of normal muscle.

The figure we report for the yield of insulin from normal dog's pancreas is low. There are several reasons for this. In those experiments in which the test animal took convulsions a much smaller dose of active substance might have been sufficient to produce the same effect on blood sugar. Convulsions were observed very frequently after administration of the pancreatic extracts. *Secondly, in this method of testing small amounts of active material yield a higher unitage than larger amounts.* Thirdly, our results are expressed in terms of a revised unit which is somewhat larger than the unit previously used. Lastly, as we were concerned in this series of experiments with the properties and amounts of insulin-like material from new sources as compared to those of insulin from pancreatic tissue, we preferred to use a tested method of preparation rather than any of the newer modifications which yield much larger quantities of active material. In this connection we suggest that the conservatism of our method may be partially responsible for the small differences observed between the yield of insulin from normal and from diabetic tissue.

The experiments upon anesthetized animals were carried out because in certain previous unpublished work we noticed that ether anesthesia appeared to lower the insulin content of the blood. The anesthetic may cause the destruction, inactivation or excretion of the material. We have not as yet attempted to ascertain whether or not the insulin content of the urine is increased after anesthesia. These results are interesting in view of the well-known influence of ether upon carbohydrate metabolism.

Very early in the course of our experiments the question arose as to whether or not this material present in various normal tissues is insulin. The material prepared from thymus glands, for example, has characteristics such as solubility in water, alcohol and other reagents, adsorption on benzoic acid, charcoal, etc., and stability, which are identical, so far as we can determine, with those of insulin from pancreatic tissue. In addition, however, there appears to be very strong physiological evidence that this active substance is insulin. In approximately 150 instances materials from tissues other than the pancreas have been administered to normal rabbits. In the great majority of these tests the active substance has markedly reduced the blood sugar level of these animals. Typical insulin convulsions were produced in many cases, and the administration of dextrose invariably relieved the animal. (We have never observed in normal rabbits any difference in the physiological effects of insulin from the normal tissues and the insulin-like material from diabetic tissues.) Material prepared from calves' thymus glands has in a number of experiments lowered the blood sugar, diminished the sugar excretion and markedly improved the general condition of completely diabetic dogs.

We are reporting our most complete experiment in table 16. It will be observed that the liver of this completely depancreatized animal contained 6 per cent of glycogen. *This experiment furnishes conclusive proof that the thymus gland of the calf contains insulin.* In another experiment, not reported here, we obtained similar results with an extract prepared from normal ox blood. From these experiments we conclude that insulin is present in tissues other than the pancreas.

As stated above, the insulin-like material in diabetic tissues appears to possess the same chemical and physiological properties as insulin. We were desirous, very early in our investigations, of testing this insulin-like material in the same manner as we have tested the insulin from thymus gland. Our difficulty was to secure sufficient active material from diabetic tissues to carry out a complete experiment upon a diabetic dog. We have attempted this, however, and in table 17 we have recorded the history of the diabetic animal from which the tissues, which were extracted for insulin, were removed. In table 18 the effect of this extract upon the blood sugar, sugar excretion, G/N ratio and general condition of a completely depancreatized dog are recorded. The marked falls in blood sugar and in the G/N ratio indicate that the active principle in these extracts is insulin. In this connection the experiment described in table 19 is of interest. This depancreatized dog lived nearly eight weeks after the removal of its pancreas and at the end of this period we were able to demonstrate small amounts of insulin-like material in its blood and other tissues. This animal received no insulin and no special dietary precautions were observed, with the exception that only a low caloric intake was permitted. The animal was weak but bright at the end of the period.

We do not believe that the benzoic acid method of preparing and the rabbit method of testing insulin are sufficiently accurate to justify an attempt to calculate from our data the distribution of insulin in the animal body. However, it appears that considerably more insulin can be extracted from the total volume of blood of an animal than from its pancreas, by the method we have employed. In a like manner it appears that the muscular tissue of an animal may contain very considerably more insulin than the pancreas. In certain experiments nearly as much insulin was obtained from the total weight of liver as from all the pancreatic tissue of the animal.

The question naturally arises as to whether the insulin which we find in tissues other than the pancreas is formed in the pancreas and carried by the blood or lymph to those tissues or whether insulin is actually manufactured in them. The demonstration of insulin-like material in yeast by Winter and Smith (6) was very quickly confirmed in our laboratories. Very soon after the publication of the results of Winter and Smith, Collip

(7), Thalheimer and Perry (8), and two of us (9), sent to press articles in which the demonstration of an insulin-like material in plants was recorded. We have been able to purify this material so that it exerts its physiological effects nearly as rapidly as insulin from pancreatic tissue (10) and have been able to show that the administration of purified plant extracts enables a depancreatized dog to store large amounts of glycogen in its liver. V. E. Levine (11), who worked in our laboratory during the past summer, has recently been able to demonstrate an insulin-like material in bacteria. In view of these results we believe that insulin may prove to be a constituent of every cell in which carbohydrate is metabolized and the islands of Langerhans to be specially developed structures which supply the active material when the demand for it is too great to be met by the insulin-producing power of the individual cell. The amounts of insulin found in tissues other than the pancreas and the presence of insulin in diabetic tissues as long as eight weeks after the removal of the pancreas support this hypothesis. The facts are, however, susceptible to other interpretations which will not be discussed in this communication.

It is surprising to find such comparatively large amounts of insulin in diabetic tisses. Very soon after the demonstration of insulin in urine by two of us (12), Fisher (13) published an article in which he stated that insulin administered per os to a normal dog might be partially recovered in the urine of the animal. We have confirmed this result and have shown that insulin administered per os to a diabetic dog may be recovered almost quantitatively in the urine of the animal. Since this material must pass through the blood stream and since it exerts no effect on the blood sugar of the animal it must exist in an inactive state in the blood. The experiments reported in this paper show that insulin is present in the tissues of a diabetic dog. This insulin may either be present in a comparatively inactive form in the diabetic tissues or some condition may exist in these tissues which inhibits the action of the insulin. A study of the insulin concentration of the blood at varying intervals after the administration of large amounts of insulin by mouth may throw light on this subject.

CONCLUSIONS

1. Insulin is present in various tissues other than the pancreas.

2. Insulin is present in reduced amounts in the tissues of the completely depancreatized dog.

3. Previous results from this laboratory, which demonstrated insulin in blood, are confirmed in this series of experiments

4. Much smaller amounts of insulin are obtained, by the method we have used, from the tissues of dogs which have been subjected to ether anesthesia, than from the tissues of normal or diabetic dogs.

We wish to express our thanks to Dr. J. G. FitzGerald and Dr. R. D. Defries for their helpful criticism and energetic support. We are indebted to Dr. V. E. Henderson of the Department of Pharmacology and Dr. F. G. Banting of the Banting-Best Chair of Medical Research for helpful criticism and facilities for physiological work during our early experiments.

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Note. As this communication was ready for press prior to the appearance of an article by J. S. Ashby in the December issue of the AMERICAN JOURNAL OF PHYSIOLOGY, we have omitted a detailed discussion of his work. Many of Ashby's results are contrary to those we have obtained.

BIBLIOGRAPHY

(1) BANTING AND BEST: Journ. Lab. and Clin. Med., 1922, 251; Idem., 1922, 464.
(2) BEST AND SCOTT: Journ. Biol. Chem. 1923, lvii, 709.
(3) MOLONEY AND FINDLAY: Journ. Biol. Chem., 1923, lvii, 359.
(4) BANTING, BEST, COLLIP AND MACLEOD: Trans. Royal Soc., Canada, 1922, xvi, 1.
(5) SHAFFER-HARTMAN: Journ. Biol. Chem., 1920, i, 349.
(6) WINTER AND SMITH: Journ. Physiol., 1922, lvii, 100.
(7) COLLIP: Proc. Soc. Exper. Biol. and Med., 1923, xx, no. 6.
(8) THALHEIMER AND PERRY: Journ. Amer. Med. Assoc., June 2, 1923, 1614.
(9) BEST AND SCOTT: Journ. Met. Research, 1923, iii, no. 1.
(10) BEST AND SCOTT: Trans. Royal Society Canada, 1923, xvii.
(11) LEVINE: Personal communication.
(12) BEST AND SCOTT: Journ. Amer. Med. Assoc., 1923, lxxxi, 382.
(13) FISHER: Journ. Amer. Med. Assoc., 1923, September 15, 920.

THE INFLUENCE OF CARBON DIOXIDE ON THE VELOCITY OF DIVISION OF MARINE EGGS

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During the summer of 1921, while examining the influence of H ion concentration on the development of marine eggs, certain of our experiments suggested that CO_2 liberated from $NaHCO_3$ normally contained in sea water exerted a marked inhibitory influence which could not be attributed to the H ions present in the solution. This supposition was confirmed by experiments on the effects of carbonic acid and H ions separately, in which the development in carbonate-free sea water of varying reactions was compared with the development in normal sea water at corresponding reactions. It was found that in carbonate-free solutions the normally fertilized eggs of *Asterias forbesii* and *Arbacia punctulata*, which were then under examination, would develop with practically normal velocity and in a normal manner at H ion concentrations as great as pH 6.0, whereas development was markedly inhibited in normal sea water at pH 7.0, and prevented at pH 6.0.

The obvious significance of this inhibitory property of CO_2 led us to engage the problem in a quantitative manner, examining the effects of definite concentrations of H_2CO_3 on the velocity of division at various H and HCO_3 ion concentrations. Some of the quantitative observations on *Arbacia* were made during the summer of 1922 and have been reported in a brief note (1). Confirmatory experiments with *Arbacia* and new experiments with *Asterias* were made during the summer of 1923. The results of these experiments will be reported completely at this time. Since the influence of H ion concentration in carbonate-free sea water on the physiological properties of *Arbacia* and *Asterias* eggs has been described in detail in another communication (2), these experiments will not be restated here.

Chemical methods. Sea water solutions having definite concentrations of H_2CO_3 and H ions were prepared by adding standard HCl to sea water alone or after the addition of definite quantities of $NaHCO_3$. Under these conditions H_2CO_3 is liberated from the $NaHCO_3$ by the added HCl, and solutions having a definite CO_2 tension can be prepared quickly, accurately and with the greatest facility for the present purposes. The

constancy of the composition of sea water in respect to total base and $NaHCO_3$ makes it possible to obtain constant results by this method. If no CO_2 is lost from the solution the concentration of H_2CO_3 (or the CO_2 tension) under these conditions will depend on the quantity of $NaHCO_3$ present and the H ion concentration of the resulting mixture.

The total carbonate content of sea water and the sea water-$NaHCO_3$ mixtures was determined by the Van Slyke method (3) which was first used in this connection by McClendon (4). The concentration of carbonates may vary under laboratory conditions from several causes; the CO_2 content may be increased by aquatic life or by absorption from air rich in CO_2; or decreased by loss of CO_2 to the air at high temperatures. But since the total available base (which is slightly in excess of the total carbonate) is extremely constant in sea water from one locality, the carbonate content can be restored to normal by shaking or otherwise allowing the sea water to come to equilibrium with air. We have found that the carbonate content (R_2CO_3) of the sea water delivered by the pumping system in the Marine Biological Laboratory during the summers of 1922 and 1923 did not vary beyond 0.00175 to 0.00185 mols per liter; the mean value, and that which will be used for all calculations in this paper, was 0.0018.[1]

The total available base (B) was determined by titrating the boiling solution in a flat dish with N/100 HCl, using bromcresol purple.

The $NaHCO_3$ solution used to increase the bicarbonate content of the sea water was, by analysis, normal in respect to total available base and 0.9 normal in respect to total carbonate.

The CO_2 tension was determined by bringing a large volume of a solution to equilibrium with a small volume of air at 20° ± 1°, and analyzing the resulting gas mixture with a Haldane apparatus. The gas samples were prepared in a side-arm suction flask fitted with a rubber stopper containing a glass stopcock, and carrying on the side arm a short piece of rubber tubing closed by a pinch cock. The total volume of the flask when closed was 255 cc. In making a determination a known quantity of sea water was placed in the flask, and after being brought to the proper temperature in the water bath, the desired quantity of acid (N/20 HCl) was added and the stopper inserted and the stopcock closed. The total volume of sea water + acid equalled 240 cc., leaving 15 cc. of air space in the flask. The flask was then inverted and shaken mechanically for one to five hours. During the shaking the side arm was connected by a

[1] McClendon's data for the total carbonate in relation to pH and total available base would indicate for pH = 8.15 and B = 0.0022, R_2CO_3 = 41 cc. N.T.P. per liter, or 0.00184. McClendon found, however, that for Woods Hole sea water B = 0.0025, so our results are hardly comparable. It may be stated that Cohn and Henderson found B = 0.0022 on Woods Hole sea water, in agreement with us. (See table 1.)

capillary tube to a manometer filled with sea water and placed immediately beside the flask. This manometer was raised or lowered from time to time so that the manometer column was kept on a level with the liquid in the flask. Without this precaution a positive pressure develops within the flask due to the liberated CO_2, which will introduce an error when the gas sample is transferred to the Haldane apparatus at atmospheric pressure by expanding the total gas volume and thereby diluting the CO_2. The capillary connection between the flask and the manometer reduces circulation between the solution in the flask and the sea water in the manometer.

The gas mixture was transferred to the gas pipette of the Haldane apparatus through an inverted Y tube, the upper arm of which carried a stopcock and a small reservoir. During the transfer the flask was returned to an upright position, the stopcock in the mouth of the flask being connected with one arm of the inverted Y tube. The other arm of the Y tube was connected with the gas pipette. The manometer was raised slightly and left in connection with the side arm of the flask to compensate the negative pressure in the flask when the gas is withdrawn. The Y tube was then filled with mercury from the gas pipette by raising the levelling bulb, and all the air expelled through the trap at the top. The trap was then closed, the stopcock leading to the flask opened and a few drops of mercury forced into the latter to remove any of the solution which might be adhering in passage. By lowering the levelling bulb, a sample of the gas mixture was then passed into the gas pipette without contamination with air and at atmospheric pressure since an equivalent volume of liquid passed from the manometer into the flask to replace the gas removed.

Several determinations of the CO_2 tensions were made on each mixture. The mean values were plotted against the quantity of acid added; from the smoothed curves obtained from these data the CO_2 tension could be read for any mixture.

The H ion concentration was determined colorimetrically using borate and phthalate standards with the phthalein indicators recommended by Clark (5). Borate standards containing NaCl to correct the salt error were prepared as recommended by McClendon (6) and standardized electrometrically. In the range covered by the phthalate standards and the indicators bromcresol purple and methyl red, the salt error is about 0.02 pH, so these standards may be used without the addition of salt. Every precaution was taken in determining the H ion concentration to prevent the loss of CO_2.

The data for the total carbonate (R_2CO_3), total base (B), and H^+ concentration for various mixtures are given in table 1, together with the partial pressure of CO_2 (P_{CO_2}) in equilibrium with these solutions at $20° \pm 1°$.

Dr. Edwin J. Cohn, in collaboration with Dr. Lawrence Henderson, made an extensive study of the bicarbonate equilibria in sea water in 1914. A condensed report of the work has been published (7), but among the data which were not published are some on the titration of sea water with N/100 HCl. Doctor Cohn and Doctor Henderson have kindly permitted us to include these data in this paper for comparison with ours. They are given in the last two columns of table 1.[2]

Biological method. Each experiment reported in this paper consists of an examination of the velocity of division of eggs placed in solutions of varying CO_2 tensions, ranging from that of sea water (ca. 0.04 per cent) to a tension required to completely prevent cell division. The experiments were performed in flat-bottom, glass-stoppered bottles having a total capacity with stopper in place of 150.5 to 152.0 cc. To minimize the error resulting from loss of CO_2 to the air space above the solution, it was necessary to keep this air space as small as possible. For this reason the mixtures of sea water and HCl were made in such a manner that the total volume would be constant. In all our experiments this total volume was 150.0 cc., leaving from 0.5 to 1.0 cc. of air space when the bottle was filled with the CO_2 solution; 150.0 cc.-x cc. of sea water or sea water-$NaHCO_3$ mixture were placed in the bottles and brought to 20°. The fertilized eggs were then added to the bottles, and x cc. of N/20 HCl added from a burette and the bottle stoppered and inverted several times to insure immediate mixing. During the experiments the bottles were

[2] The observed values of the H ion concentration and CO_2 tension of sea water to which HCl has been added have been compared with corresponding values calculated on the basis of the Henderson-Hasselbach equation. The carbonate system is profoundly influenced by the neutral salts of sea water which presumably decrease the dissociation of $NaHCO_3$. The correction for this effect is customarily expressed in the apparent dissociation constant of the acid; thus $K' = \frac{Ka}{\gamma}$, where Ka is the dissociation constant in distilled water, K' is the apparent dissociation constant in the salt solution and γ the degree of dissociation of the salt. (The second dissociation may be neglected within the range with which we are concerned.) If the titration curve of sea water + HCl is calculated from the data of Henderson and Cohn, making $B = 0.0022$, and a similar curve calculated from our data on the CO_2 tension, using a value of 31.2 for C in $H_2CO_3 = P_{CO_2}/C$, these curves agree in indicating a value of K' of 4.47×10^{-7} (pK = 6.35). $C = 44/1.7909 \times 0.787$ where 44 = M.W. CO_2, 1.7909 = d. at 20°, and 0.787 = absorption coefficient in sea water (7). Seylor and Lloyd (8) have recently found a value for γ in 0.5 M salt solutions of 0.64. Kendall (9) has recently redetermined Ka for carbonic acid, and gives the figures 3.12×10^{-7} at 18° and 3.50×10^{-7} at 25°. A value of $Ka = 3.25 \times 10^{-7}$ at 20° coupled with $\gamma = 0.64$ gives $K' = 5.1 \times 10^{-7}$. This value agrees satisfactorily with the above data.

The observed and calculated titration curves deviate appreciably between pH 6.7 and 7.7, indicating the presence in sea water of a weak acid other than H_2CO_3 (7).

kept in a bath at 19 to 21°. Since the air space in each bottle was less than 1 per cent of the total volume of liquid, only a small fraction of the total free CO_2 was present in this space, and therefore the loss of CO_2 on removing the stopper to obtain samples of eggs for counting was slight.

TABLE 1

MIXTURE	R_2CO_3	B	N/20 HCl PER 100 cc. MIX.	P_{CO_2} FROM SMOOTH CURVE	pH FROM SMOOTH CURVE	TITRATION OF 100 cc. SEA WATER WITH N/100 HCl (FROM COHN AND HENDERSON)	
						HCl	pH
			cc.			cc.	
Seawater	0.0018	0.0022	0.5	0.005	7.5	0.5	7.95
			1.0	0.0105	7.0	1.0	7.82
			2.0	0.0244	6.42	1.5	7.70
			3.0	0.038	5.96	2.0	7.60
			4.0	0.0539	5.00	2.5	7.50
998 cc. sea water, 2 cc. $NaHCO_3$	0.0036	0.0042	1.0	0.0100	7.48	3.0	7.41
			2.0	0.0235	6.95	3.5	7.31
			3.0	0.038	6.58	4.0	7.20
			4.0	0.050	6.36	4.5	7.10
			5.0	0.065	6.14	5.0	7.00
			6.0	0.080	5.87	6.0	6.85
996 cc. sea water, 4 cc. $NaHCO_3$	0.0054	0.0062	1.0	0.0110	7.65	7.0	6.70
			2.0	0.0225	7.29	8.0	6.58
			3.0	0.0355	6.94	9.0	6.50
			4.0	0.0490	6.66	10.0	6.42
			6.0	0.0790	6.35	11.0	6.35
994 cc. sea water, 6 cc. $NaHCO_3$	0.0072	0.0082	1.0	0.0105	7.75	12.0	6.27
			2.0	0.0215	7.47	13.0	6.17
			3.0	0.0345	7.20	14.0	6.08
			4.0	0.048	6.93	15.0	6.00
			6.0	0.074	6.56	16.0	5.90
992 cc. sea water, 8 cc. $NaHCO_3$	0.0090	0.0102	2.0	0.0210	7.56	17.0	5.78
			3.0	0.0330	7.37	18.0	5.64
			4.0	0.0465	7.13	19.0	5.44
			5.0	0.0610	6.93	20.0	5.23
			6.0	0.0750	6.76	21.0	4.96
			7.0	0.0895	6.62	22.0	4.48

On several occasions determinations of the total CO_2 content and the pH were made on the solutions during the experiment, but since the removal of a large amount of liquid introduced an appreciable error in the CO_2 tension, it was impossible to do this when the subsequent development of

the eggs was under observation. A sufficient number of determinations was made, however, to show that the quantity of CO_2 liberated by the growing eggs during the course of the experiments was inappreciable in comparison with the concentrations present in the solutions.

The experiments reported in this paper were performed with the eggs of the sea urchin, *Arbacia punctulata*, and the star fish, *Asterias forbesii*. The eggs were obtained from the ovaries in the usual manner and inseminated with dilute fresh sperm. Where it was desired to place eggs in the CO_2 solutions immediately, they were allowed to settle to the bottom of the finger-bowls and were then transferred with a pipette to centrifuge tubes. They were then gently centrifuged, the supernatant sea water poured off and equal quantities, usually 3 to 8 drops, of the heavy suspension obtained from the bottom of the centrifuge tubes added to each of the bottles, already prepared and in the bath. The HCl was then added, the solution thoroughly mixed and the bottles returned to the bath. The bottles were usually shaken gently every ten minutes during the experiment, except just prior to sampling.

Controls were always included which consisted of portions of the same lot of eggs placed in bottles containing sea water or sea water-$NaHCO_3$ solutions. In a number of instances controls were also kept in open bowls at the same temperature. These controls showed that the eggs in the bottles did not suffer because of oxygen deficiency and that the CO_2 liberated from the growing eggs did not influence the results. Both of these factors may become important if large quantities of eggs are placed in smaller quantities of liquid, but they are wholly absent in our experiments where comparatively few eggs are placed in 150 cc. of liquid.

The determination of the rate of cell division. At regular intervals after the eggs had begun to divide small samples (0.25 to 0.5 cc.) were removed with a capillary pipette from the bottom of each bottle. These samples were fixed at once; *Arbacia* eggs were added to 5 cc. of sea water containing 1 drop of formalin, and *Asterias* eggs were added to 5 cc. of a 1-1000 bichloride solution in sea water. This treatment instantly stops all processes of cell division and preserves the eggs so that their appearance remains essentially unchanged for 24 hours. The samples could therefore be examined at leisure and quantitative determinations made on the extent of cell division.

Careful counts were made on each sample, noting the number of eggs which were undivided and the number which were in each stage of division. The number of eggs in the 2-cell stage was multiplied by one, the number in the 4-cell stage by two, the number in the 8-cell stage by 3, etc., thus obtaining the total number of divisions. This figure was then divided by the total number of eggs counted to obtain the average number of divisions per egg in each sample. This figure is an arithmetic index of the velocity of development.

All eggs which were immature or which had no membranes were neglected in making the counts. These eggs were obviously never fertilized and would, therefore, never divide under any conditions. Except in special cases all membraned (or fertilized) eggs were included because normally every egg which fertilizes will divide.

The influence of CO_2 on Arbacia eggs. The effect of increasing CO_2 tension is to decrease the rate of division, and in consequence to decrease the degree of development at any interval of time after transfer to the CO_2 solution. The general relations between rate of division and CO_2 tension can be readily seen by plotting the average divisions per egg as abscissae and the CO_2 tension as ordinates; observations made at various intervals after insemination can then be connected by contour lines.

The results of a typical experiment with *Arbacia* eggs exposed to various CO_2 tensions obtained by adding HCl to sea water are given in figure 1. In this experiment the eggs were placed in eight solutions five minutes after insemination; sea water in an open bowl, sea water in a stoppered bottle, and in sea water-HCl mixtures having CO_2 tensions of 0.8, 1.63, 2.5, 3.2, 3.8 and 4.2 per cent. Samples were taken from each lot 90 minutes after insemination and every 20 minutes thereafter. The eggs in the bottle of sea water (circles, fig. 1) divided just as rapidly as those in the open bowl (dots, fig. 1) which showed that being inclosed in the bottles was in no way unfavorable to development. As the CO_2 tension increased in the successive bottles the rate of division was reduced, until at a critical CO_2 tension of 3.8 per cent division was completely prevented. The crosses and dotted line show the averages of the six observations.

We have found that this critical CO_2 tension (i.e., which completely prevents cell division) can be determined more accurately by extrapolating the contour lines than by actual observation. At CO_2 tensions slightly lower and slightly greater than this critical value there is a tendency for some eggs to divide irregularly, and consequently the actual condition of the eggs in this region will not indicate the critical CO_2 tension more closely than $\pm$ 0.2 of 1 per cent. But by extrapolating the contour lines it may be obtained to $\pm$ 0.1 of 1 per cent.

The shape of the contour lines in different experiments, though in general resembling those shown in figure 1, shows considerable variation. In the experiments cited here the rate of division is not an inverse function of P_{CO_2}, though it approximates this relation very closely. The conditions that determine this function cannot be stated but appear to be variable and dependent on physiological rather than environmental circumstances. It is a singular fact that all experiments on *Arbacia* performed in the summer of 1923 showed a curvilinear relation between developmental velocity and P_{CO_2} similar to that illustrated in figures 1 and 2, while some three dozen experiments performed during the summer of 1922 all showed

a strict linear relation. In none of these earlier experiments was there a sufficient departure from this linear relation to suggest that it was not a permanent condition in the action of CO_2.

The influence of CO_2 differs in no essential respects if the eggs are transferred to the CO_2 solutions immediately after insemination or after they have begun to divide. This fact is illustrated in figure 2. This experiment is a mate to the experiment illustrated in figure 1 and was

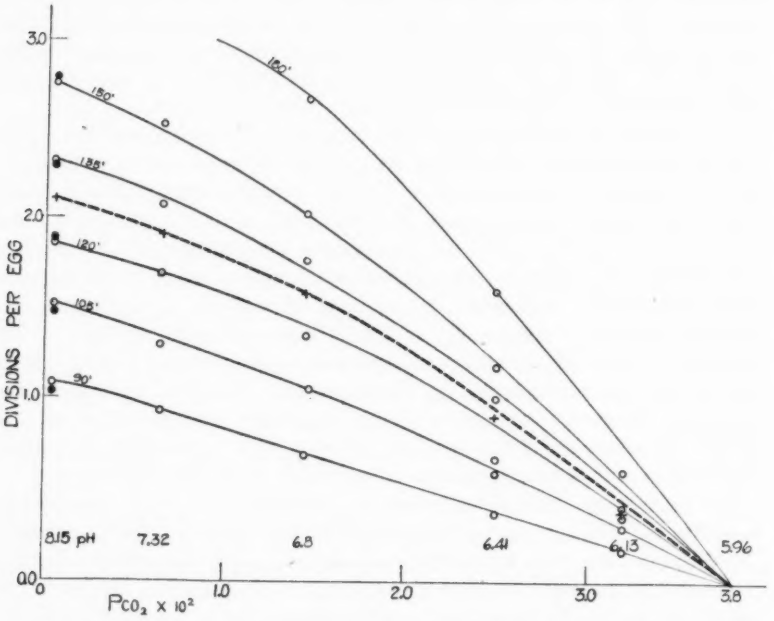

Fig. 1. The influence of increasing CO_2 tension on the development of normally fertilized *Arbacia* eggs when transferred to the CO_2 solutions 10 minutes after insemination. The figures on the contour lines show the number of minutes after insemination at which samples were taken. The dotted line shows the mean of all the observations.

performed simultaneously with all the conditions the same except that the eggs were left in sea water until a large fraction had reached the two cell stage. This was 60 minutes after insemination. They were then placed in the CO_2 solutions, and samples taken at intervals of 15 minutes. Above the critical CO_2 tension the eggs which had already divided remained in the two-cell stage; the rest remained undivided. The critical CO_2 tension in this case is the same as when the eggs are transferred immediately after insemination, namely 3.8 per cent.

The influence of CO_2 on Asterias eggs. The CO_2 tension required to completely repress the division of *Asterias* eggs is exactly the same that is required to repress the division of *Arbacia* eggs. The effects of CO_2 are qualitatively and quantitatively much the same except that the shape of the contour lines varies considerably with the time of transfer to the CO_2 solutions. As the egg approaches the two cell stage, low CO_2 tensions become less and less effective in retarding division and ultimately may accelerate it. This is illustrated by three simultaneous experiments in

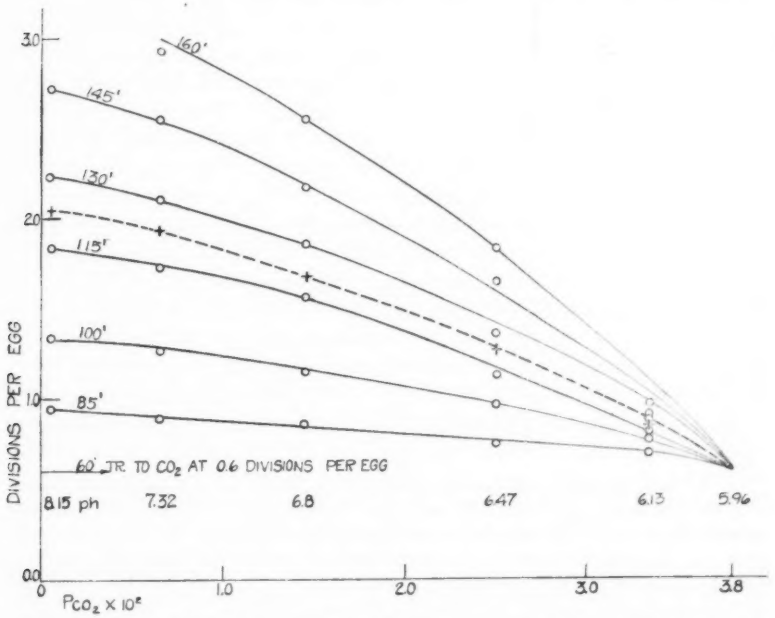

Fig. 2. Same as figure 1 except that *Arbacia* eggs were transferred to the CO_2 solutions 60 minutes after insemination, at which time 60 per cent of the eggs were in the 2-cell stage.

figure 3. In the first of these experiments the *Asterias* eggs were transferred to the CO_2 solutions 5 minutes, in the second 50 minutes and in the third 100 minutes after insemination. In the last case 50 per cent of the eggs were already in the two cell stage. In each case the development was followed through two cleavages only, so that the immediate effects of the CO_2 solutions might be more evident. Five determinations of the number of divisions per egg were made at 15 minute intervals. These five values were then averaged and the mean expressed as per cent of the corresponding mean for sea water. In this way the curves are given a com-

mon origin. (Data expressed in this manner are indicated in the figures as "per cent development, sea water = 100 per cent".)

The inhibitive action of low CO_2 tensions decreases as the egg nears the first cleavage (about 100 minutes after insemination) and ultimately the CO_2 serves to increase, rather than to decrease the velocity of cell division. This stimulation is not always observed, and when present does not persist for more than two or three cleavages. Ultimately the advanced cultures fall back more or less in line with the controls and keep abreast

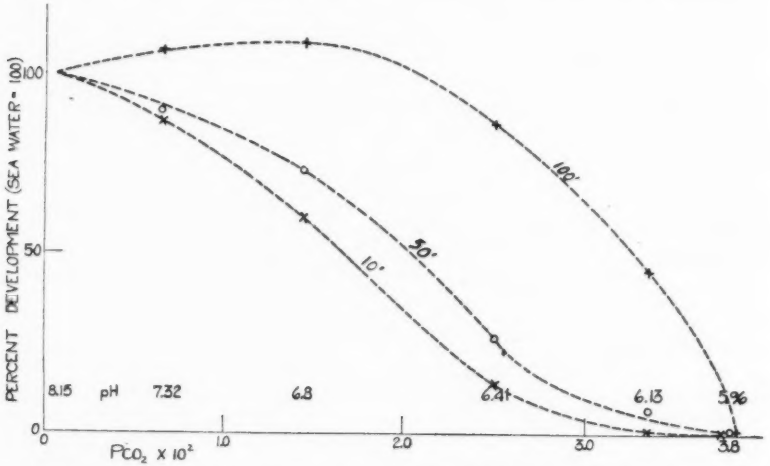

Fig. 3. The influence of increasing CO_2 tension on the development of normally fertilized *Asterias* eggs. Each of the three curves represents the mean results obtained from an experiment similar to those shown in figures 1 and 2. The development is expressed in terms of the mean development in sea water as 100 per cent, so that the curves will have a common origin. The figures on the contour lines show the number of minutes after insemination at which the eggs were transferred to the CO_2 solutions. As the *Asterias* egg approaches the first cleavage plane it develops an increased resistance to low CO_2 tensions, but the critical tension remains the same. The critical tension for *Asterias* is the same as for *Arbacia*, namely, 3.8 per cent.

with them to the swimmer stage. The variation in resistance of *Asterias* to low CO_2 tensions seems to be confined to the pre-cleavage period; during this period the developmental processes are retarded uniformly by low as well as high CO_2 tensions. After the egg has reached the 2-cell stage it is no longer affected by low CO_2 tensions, though, significantly, the critical CO_2 tension remains the same. This change in resistance to CO_2 during the pre-cleavage period recalls a similar change in the resistance of *Asterias* eggs to alkali recently described by us (2). The latter change is in the same

direction; as the egg approaches the first cleavage, the resistance to alkali becomes permanently increased.

The influence of H and HCO_3 ions on the inhibitive power of definite CO_2 tensions. The H ion concentrations of sea water-HCl mixtures used in the above experiments are given in the margin. We have previously shown that variations in the H ion concentration of CO_2-free sea water within the limits involved here do not materially influence the rate of cell division of *Arbacia* and *Asterias* eggs (2). *Arbacia* eggs divide with undiminished velocity and without any abnormality at pH 6.0, and the rate of division in *Asterias* eggs is barely reduced by 10 per cent at the same reaction. Therefore it might be supposed that the complete repression of cell division in the presence of carbonates is due solely to the action of free CO_2, and that the H ions in the solutions play no part in this action. This point is open to direct examination since the H ion concentration can be varied independently of the CO_2 tension by varying the $NaHCO_3$ content of the sea water: solutions of the same CO_2 tension can be obtained in which the H ion concentration varies inversely as the $NaHCO_3$ content.

We have examined the influence of CO_2 at different H ion concentrations by this method. The mixtures were prepared by adding a definite quantity of a normal $NaHCO_3$ solution to sea water and acidifying the resulting mixtures as in the previous experiments.[3]

The results obtained with three such mixtures on both *Arbacia* and *Asterias* eggs are given in figures 4 and 5, and summarized in table 2. In the figures the per cent development in terms of the development in sea water as 100 is plotted against the CO_2 tension. Each contour line represents the mean values obtained from one experiment with each mixture. The data on the contour lines give the total carbonate content of each mixture, and those at the bottom of the graph the pH at the critical CO_2 tension.

It will be observed that the effectiveness of CO_2 in repressing cell division diminishes uniformly as the total bicarbonate content of the solution increases. The rate of change is the same for *Asterias* eggs as for *Arbacia* eggs, so that the critical CO_2 tension of any mixture is the same for both species.

The varying effectiveness of CO_2 in sea water-$NaHCO_3$ mixtures shows that the physiological action of CO_2 does not depend exclusively on the free carbonic acid, but involves other substances present in these mixtures. The question of what factor or factors are responsible for this variation can only be answered at present by the indirect process of elimination.

[3] Attempts were made to use sea water with less than its normal content of $NaHCO_3$, but the acidity of such solutions complicates the results. Below pH 5.4–5.6 the influence of CO_2 and H ion concentration become additive, and consequently the relations existing above pH 5.8 no longer hold.

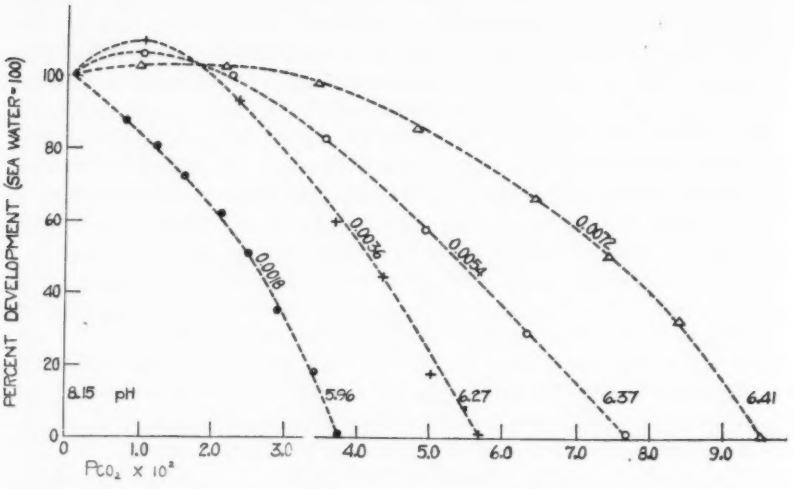

Fig. 4. The influence of increasing CO_2 tension on the development of *Arbacia* in the presence of increasing quantities of $NaHCO_3$. Each curve is the mean of several experiments. The figures on the contour line show the concentration of $NaHCO_3$ in the original sea water-$NaHCO_3$ mixture.

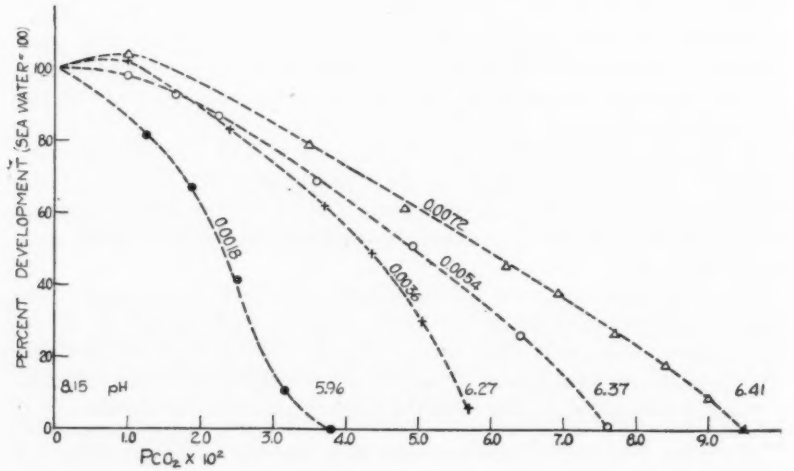

Fig. 5. Same as figure 4 for *Asterias* eggs.

The species involved in carbonate equilibria are (other than H_2CO_3 and CO_2) $BHCO_3$ or HCO_3^-, $CO_3^=$, H^+, OH^-, B^+ or Na^+, and A^- or Cl^-. Since sea water is 0.5 normal in respect to B^+ and A^-, the actual variations in the concentrations of these ions in the several mixtures is relatively very small; these ions may therefore be neglected. The actual concentration of $CO_3^=$ is so small in the range with which we are concerned (pH 6.0 to pH 6.5) that this ion may also be neglected. There remains the $H^+ \times OH$ balance and $BHCO_3$. ($BHCO_3$ is used here to indicate undissociated salt + HCO_3^-; at the concentrations involved here the real value of HCO_3^- will be essentially determined by the degree of dissociation of the salt, γ, and this can be neglected for our present purposes.)

TABLE 2

ORIGINAL MIXTURES		CRITICAL MIXTURES		N/20 HCl 100 cc. MIX.	$P_{CO_2} \times 10^2$	H_2CO_3* $\times 10^4$	$BHCO_3$ $\times 10^4$	$H \times 10^7$	pH
$R_2CO_3 \times 10^4$	$B \times 10^4$	$R_2CO_3 \times 10^4$	$B \times 10^4$						
(*Arbacia*, 1922)									
				cc.					
18	22	17.5	21.3	3.0	3.8	12.2	5.3	11.0	5.96
38	44.5	36.2	42.4	4.65	6.0	19.2	17.0	5.25	6.28
59	67	55.3	62.1	6.25	8.2	26.2	29.1	4.17	6.38
79	89.5	72.8	82.5	7.85	10.2	32.6	40.2	3.80	6.42
(*Arbacia* and *Asterias*, 1923)									
18	22	17.5	21.3	3.0	3.8	12.2	5.3	11.0	5.96
36	42	34.4	40.1	4.45	5.7	18.2	16.2	5.38	6.27
54	62	50.9	58.5	5.8	7.6	24.4	26.5	4.27	6.37
72	82	66.8	76.0	7.25	9.5	30.4	36.4	3.90	6.41

* Calculated on assumption that the absorption coefficient of CO_2 is not changed by the change in salinity caused by the addition of HCl.

The rate at which the concentrations of $BHCO_3$ and of H ions change in relation to the critical CO_2 tensions of these various mixtures is shown in figure 6. (The concentration of H_2CO_3 ($= PCO_2/C$)[2] rather than the CO_2 tension has been used to express the critical concentration of free CO_2.) The curve relating H_2CO_3 to the H ion concentration is hyperbolic and is, within the limits of observation, approaching with a rapidly diminishing decrement a lower limit of 2 or 3×10^{-7}. But the critical concentration of H_2CO_3 is increasing by essentially equal increments with equal increments of the total carbonate content, and it has the appearance of continuing to increase in this manner for some time if the range of observation could be extended. It is very unlikely that this regular increment in H_2CO_3 could be a direct function of the rapidly diminishing decrement in

H ion concentration. On the other hand, the curve relating H_2CO_3 to $BHCO_3$ is, within the limits of observation, a straight line: and the increment in $BHCO_3$ is of the same order of magnitude (2:1) as that of H_2CO_3. From this fact it may reasonably be inferred that the decrease in effectiveness of CO_2 with increasing total carbonate is due to the increase in the concentration of either undissociated $NaHCO_3$, HCO_3^- ions, or both in the sea water.

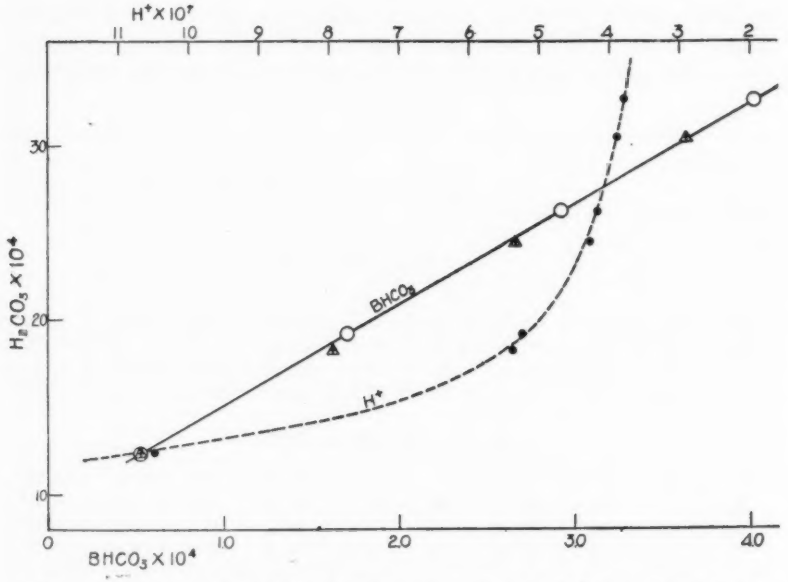

Fig. 6. The rate at which the critical concentration of H_2CO_3 increases in relation to the concentration of $BHCO_3$ and of H ions. The circles show the results obtained with *Arbacia* eggs in 1922, and the triangles and crosses the results obtained with *Asterias* and *Arbacia* eggs in 1923.

The reason for the dependence of the action of CO_2 on $BHCO_3$ cannot be stated with any surety, but a provisional hypothesis can be based on the assumption that the cell is, under certain conditions at least, permeable to $BHCO_3$.[4] For it follows from the relation $H^+ = K \frac{H_2CO_3}{BHCO_3}$, that

[4] M. M. Brooks (10) has recently found that when *Valonia* cells are placed in sea water to which $NaHCO_3$ or $KHCO_3$ has been added, the H ion concentration of the cell sap first increases rapidly, due to the penetration of CO_2, and then slowly decreases. The decrease continues until the sap is more alkaline than normal, indicating the penetration of $BHCO_3$.

for the same intracellular concentration of H_2CO_3 the H ion concentration will vary inversely as the intracellular concentration of $BHCO_3$. If we suppose that an increase in CO_2 tension within the cell leads to a migration of HCO_3 ions (with possibly a simultaneous migration of Na ions) into the cell, then the final intracellular equilibrium will be determined not only by the CO_2 tension of the sea water but also by the availability of HCO_3 ions.

In this view we can, from the available data, relate the intracellular and extracellular equilibrium in respect to $BHCO_3$. To do this it will be necessary to make two assumptions:

1. That conditions of equal repression of cell division represent conditions of equal intracellular H ion concentration. Putting intracellular concentrations in round brackets, we may write

$$(H^+)_1 = (H^+)_2 = (H^+)_3 \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

2. That under conditions of complete repression of cell division, the CO_2 tension within the cell is equal to the CO_2 tension outside. Since $P_{CO_2}/C = H_2CO_3$, then the intracellular concentration of H_2CO_3 would equal the extracellular concentration. Using square brackets to denote extracellular concentrations, we may write

$$(H_2CO_3)_1 = [H_2CO_3]_1$$

or

$$(H_2CO_3)_2 = [H_2CO_3]_2 \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

But

$$H^+ = K\frac{H_2CO_3}{BHCO_3} \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Therefore

$$K\frac{(H_2CO_3)_1}{(BHCO_3)_1} = K\frac{(H_2CO_3)_2}{(BHCO_3)_2} \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

The equation for the experimental curve relating $[H_2CO_3]$ to $[BHCO_3]$ is

$$[H_2CO_3] = c([BHCO_3] + 0.0017) \quad (\text{where } c = 0.56) \ldots\ldots (5)$$

Substituting for (H_2CO_3) in (4) by (2) and (5),

$$\frac{(BHCO_3)_1}{(BHCO_3)_2} = \frac{[BHCO_3]_1 + 0.0017}{[BHCO_3]_2 + 0.0017} \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

That is, the linear relation between H_2CO_3 and $BHCO_3$ illustrated in figure 6 affords evidence, under the assumptions made above, that the increment in the intracellular concentration of $BHCO_3$ is proportional to the increment in the extracellular concentration, and the former is therefore equal to the latter multiplied by a constant, β.

The total available base in the cell is, however, obviously not equal to $[BHCO_3]$, for in this case all physiologically equivalent solutions would have the same H ion concentration, which is not true. That the total intracellular available base is greater than $[BHCO_3]$ is indicated by the fact that a considerable concentration of H_2CO_3 is required (by extrapolation) to repress cell division when $[BHCO_3] = 0$. (The significance of this fact is not clear; it may denote available base combined with acids other than carbonic.) We may, therefore, write (6) in the form

$$(BHCO_3) = \alpha + \beta[BHCO_3] \quad (7)$$

Substituting in (4) for $BHCO_3$

$$\frac{(H_2CO_3)_1}{\alpha + \beta[BHCO_3]_1} = \frac{(H_2CO_3)_2}{\alpha + \beta[BHCO_3]_2} = \frac{(H_2CO_3)_3}{\alpha + \beta[BHCO_3]_3} \quad (8)$$

Substituting observed values in (8), it follows that

$$\alpha = 0.0017\beta \quad (9)$$

Through the actual relation between H_2CO_3 and $BHCO_3$ leads, on the foregoing assumptions, to the deduction that the intracellular concentration of $BHCO_3$ increases in proportion to the extracellular concentration, the data afford us no means of determining the actual concentration of $BHCO_3$ in the cell, for any values may be substituted for α and β so long as they fulfil equation 9.

The above relations are such as to acquire plausibility when one considers the probable H ion concentration compatible with cell division. For example, if $\beta = 0.56$, (pH) = 6.35; if $\beta = 1.0$, (pH) = 6.6; if $\beta = 2.0$, (pH) = 6.9.[5]

Discussion. That CO_2 might play a rôle in physiological processes other than that of a passive end product was first suggested by the work of Haldane and Priestley (11) on the regulation of the mammalian respiratory center. How much of the action in this case is due to CO_2 and how much to H or HCO_3 ions is still an unsettled question. The subject has recently been reviewed by Wilson (12). The importance of carbonates in other respects was subsequently pointed out by Lawrence Henderson (13). Henderson emphasized the fact that the precise degree of dissociation of carbonic acid places the buffer capacity of its salts within the range of physiological reaction, and to the fact that the volatility of the anhydride CO_2 not only enables the organism to effect a precise and rapid adjustment of the reaction of its tissues, but simultaneously permits the excretion of 99 per cent of the acid end products of metabolism.

The fact that CO_2 could penetrate living cells was recognized by Pfeffer (14) and earlier physiologists as a necessary condition of its constant

[5] On the assumption that (C) = [C] and (pK) = [pK] = 6.35.

respiration in both animal and plant tissues. But that its penetrating power is extraordinary in comparison with other acids has been recently shown by Jacobs. By using the petals of *Symphytum peregrinum*, which contain a pigment having the properties of an indicator, Jacobs has found that sufficient quantities of CO_2 to produce a color change penetrate in one to two minutes as compared with fifteen to thirty minutes required for butyric, acetic and salicylic acids (15). When *Asterias* eggs vitally stained with neutral red are placed in slightly alkaline bicarbonate solutions, they acquire an increased acid reaction in consequence of the penetration of the CO_2 present in the solutions in small quantities (16). For the same reasons neutral or slightly alkaline bicarbonate solutions have an acid taste (17). Jacobs compared the toxicity of various acids at the same H ion concentration for tadpoles and protozoa, and found that solutions containing CO_2 were incomparably more toxic. In certain protozoa an increased H ion concentration (HCl) paralyzes the cilia first and only slowly paralyzes the vacuoles and produces coagulation and swelling of the cytoplasm. Acid solutions of CO_2, on the other hand, arrest the vacuoles and produce disintegrative changes in the cytoplasm before the cilia are sufficiently affected to bring about cessation of locomotion (18). Jacobs concludes that the action of CO_2 solutions is not attributable to their H ion concentration, but to the relatively large amount of the undissociated acid (or dissolved CO_2) which exists in these solutions by virtue of the weakness of the acid and the consequent hydrolysis of its salts (16), (17).

The penetrating power of CO_2 is so remarkable that it excites interest for, in fact, it seems to penetrate living tissues with greater facility than any other known substance, including water. It seems to us from several considerations that this penetrating power is attributable not to the acid H_2CO_3, but to the anhydrous dissolved CO_2.

It is commonly assumed in considering carbonate equilibria that all dissolved CO_2 exists as H_2CO_3. Thus the ionization constant of H_2CO_3 is based on this assumption. There is good evidence that this is not so, however, but that only a small portion of the dissolved CO_2 is hydrated. From indirect considerations Walker and Cormack (19) concluded that the ratio $\frac{H_2CO_3}{CO_2}$ is greater than 2.0. There has as yet been no direct method devised whereby we can distinguish between H_2CO_3 and dissolved CO_2, but an indirect estimate of the true strength of carbonic acid has been made by Thiel and Strohecker (20). This method is based on the familiar observation that when an equivalent quantity of a CO_2 solution is rapidly added to an alkaline solution containing an indicator, the alkaline color of the indicator persists for a considerable time. In the case of a mineral acid or acetic acid, the time required for complete neutralization depends only on the time of mixing, which Thiel and Strohecker

were able to reduce to 0.4 second. They attributed the delay due to the reaction $CO_2+H_2O \rightarrow H_2CO_3$. By using smaller and smaller quantities of alkali the neutralization time was progressively shortened. It was estimated from their experiments that in a 0.00812 molar normal CO_2 solution at 4°, only 0.67 per cent of the total concentration is actually carbonic acid, and therefore available for instantaneous neutralization. From this figure they calculated the true dissociation constant to be 5.0×10^{-4}, in contrast to the apparent value of 3.04×10^{-7} (18°). This result is in agreement with the chemical structure of carbonic acid. Since the substitution of OH for H in acetic and propionic acids produces an increase in the dissociation constant it would be expected that carbonic acid, which is hydroxy-formic acid, would be stronger than formic acid ($k_a = 2.1 \times 10^{-4}$).

The exact ratio of CO_2 to H_2CO_3 is not a matter of great moment; if we recognize that a large fraction of the dissolved CO_2 exists as CO_2 and not as H_2CO_3, the physiological aspects of the problem are materially altered. It is not possible to study the acid H_2CO_3 and the anhydride CO_2 separately in respect to their power to penetrate living cells. But there is no reason to believe that H_2CO_3 possesses greater penetrating power than acetic or even lactic acid. The penetrating power of the aliphatic acids decreases in general with descent of the series; thus caproic > valeric > propionic > acetic (21); the introduction of a hydroxyl group diminishes as a rule the activity of any acid; thus (propionic >) acetic > lactic (21), (22), (25). Since carbonic acid is hydroxyformic acid, we would expect it to have no more, if not less, penetrating power than lactic acid or glycollic acid.

On the other hand CO_2 is, unlike the lower aliphatic acids and therefore presumably H_2CO_3, capable of penetrating artificial and natural membranes with the greatest facility. Krogh (23) found that CO_2 penetrated animal tissues thirty-five times as fast as O_2, which Harvey has recently shown penetrates with apparent freedom at all times (24). It seems highly improbable that this great penetrating power is a property of the undissociated acid, H_2CO_3. Since the anhydride is much more soluble in nearly all organic liquids than in water, and since it is chemically inert, it is probable that by these virtues it is able, like other non-electrolytes, to penetrate the cell without passing through channels which involve ionic equilibria.

It is a noteworthy fact that carbonic acid is the only naturally occurring acid which exists in aqueous solution in equilibrium with an appreciable concentration of its anhydride. This duality, coupled with the physical properties of the anhydride on the one hand and the chemical properties of the acid on the other, appears to endow CO_2 with its exceptional mobility in living tissues.

The inhibitive action of CO_2 on the division of *Arbacia* and *Asterias* eggs presents several significant physiological aspects which will be discussed in a subsequent communication.

SUMMARY

The early development of the normally fertilized eggs of *Asterias forbesii* and *Arbacia punctulata* has been followed by quantitative methods under conditions of varying CO_2 tension, H and HCO_3 ion concentrations in sea water.

Increasing the CO_2 tension reduces the velocity of cell division. At a definite and constant tension, which is the same in both species, cell division is completely repressed.

The effectiveness of CO_2 in repressing cell division decreases as the total bicarbonate content of the sea water increases. The provisional hypothesis is advanced that this is due to the penetration of $BHCO_3$ into the cell. On the further assumptions that the intracellular CO_2 tension always equals the extracellular CO_2 tension, and that conditions of equal repression cell division represent conditions of equal intracellular H ion concentration, it is shown that the intracellular concentration of $BHCO_3$ increases in simple proportion to the extracellular concentration.

It is suggested that the great penetrating power possessed by carbonic acid is attributable to the dissolved gas CO_2, rather than to H_2CO_3.

We are indebted to Mabel T. Studebaker for the statistical work in the experiments recorded in this paper.

BIBLIOGRAPHY

(1) Clowes and Smith: Journ. Biol. Chem., 1922, l, iv.
(2) Smith and Clowes: Biol. Bull., in press.
(3) Van Slyke: Journ. Biol. Chem., 1917, xxx, 347.
(4) McClendon: Journ. Biol. Chem., 1917, xxx, 259.
(5) Clark: The determination of hydrogen ions. Baltimore, 1920.
(6) McClendon: Journ. Biol. Chem., 1917, xxx, 265.
(7) Henderson and Cohn: Proc. Nat. Acad. Sci., 1916, ii, 618.
(8) Seylor and Lloyd: Journ. Chem. Soc., 1917, cxi, 138.
(9) Kendall: Journ. Amer. Chem. Soc., 1916, xxxviii, 1480.
(10) Brooks: U. S. Pub. Health Repts., 1923, 1470, no. 846.
(11) Haldane and Priestley: Journ. Physiol., 1905, xxxii, 225.
(12) Wilson: Physiol. Rev., 1923, iii, 295.
(13) Henderson: The fitness of the environment. New York, 1913.
(14) Pfeffer: Physiology of plants (Ewart's translation). Oxford, 1900.
(15) Jacobs: This Journal, 1920, liii, 457.
(16) Jacobs: Journ. Gen. Physiol., 1922-23, v, 181.
(17) Jacobs: This Journal, 1920, li, 321.
(18) Jacobs: Journ. Exper. Zoöl., 1912, xii, 519.

(19) WALKER AND CORMACK: Journ. Chem. Soc., 1900, lxxvii, 5.
(20) THIEL AND STROHECKER: Ber., 1914, xlvii, 945.
(21) CROZIER: This Journal, 1918, xlv, 315; Journ. Biol. Chem., 1916, xxiv, 443.
(22) HAAS: Journ. Biol. Chem., 1916, xxvii, 225.
(23) KROGH: Journ. Physiol., 1919, lii, 391.
(24) HARVEY: Journ. Gen. Physiol., 1922, v, 215.
(25) LOEB: Artificial parthenogenesis and fertilization. Chicago, 1913.

HAIR BALL FORMATION IN RATS IN RELATION TO FOOD CONSISTENCY

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Since the origin of the "standard casein" diet by Osborne and Mendel (1), many laboratories have adopted this feeding device for rats to prevent scattering. Growth on such diets has proven most satisfactory and the convenience and ease of modification has made the procedure popular for research workers. The chief criticism has been due to the recent observations of several laboratories that rats do not reproduce normally and are often sterile on such diets. Experiments along this line in our laboratory have revealed a new and unexpected difficulty which may be of interest to other workers using paste food diets.

Rats from the same colony fed on our stock diet of mixed dried foods (bread, oats, corn, milk powder, greens) furnished a basis of comparison for the observations herein reported. For two years or more occasional hair balls had been noted in the stomachs of certain rats but no particular significance was attached to the finding until last summer, when one of our number (Carlson) noted that hair balls were never found in stock rats. We then began to examine carefully for hair balls in every animal that died or was killed. The striking results are recorded in the following table.

Of the rats receiving the paste foods made up with 25 to 27 per cent of fat, 90 per cent have shown either definitely-formed hair balls or wads of hair in the pyloric end of the stomach. Those rats which have been on the stock food or the other diets which are dry enough to be crumbly have been consistently free from hair balls, an observation which is convincing because of the large number examined. In addition, rats on the milk or cereal and milk diets are equally free from hair balls. It is quite possible that the presence of hair balls may have been overlooked by other workers even where presumably thorough autopsy examinations have been made because the stomach contents would not ordinarily be examined.

In analyzing the possible relationship between the food consistency and the occurrence of hair balls, it was observed that since the food is usually left in the cage during the greater part of the day, chance is given for considerable hair to accumulate on the sticky surface of the food. Even more probable is the tendency for rats in licking themselves to swallow

some hair, and then when the food is eaten the hairs become coated with the fat which makes them stick together and gradually form hard packed balls. Some are smooth and shiny and feel like hard rubber; others seem less compact and show the hairs on the surface; still others are hard but rough, due possibly to some foreign particles becoming entangled in the hairs. Microscopic examination of a cross-section of one of the rubbery balls showed a less dense center of tangled hairs surrounded by more compact material toward the surface. Chemical analysis showed a small percentage of fat. They are practically always found in the pyloric end of the

TABLE 1

	NUMBER OF RATS ON DIET	HAIR BALLS PRESENT	HAIR BALLS ABSENT
Paste foods:			
Standard with 5 per cent agar	35	31	4
Standard	3	3	
Standard (crisco replacing butter)	3	3	
Modification with 18 per cent egg white	2	2	
Modification with 50 per cent casein	1	1	
Modification with 50 per cent meat protein	2	2	
Modification with 20–40 per cent cream of wheat	4	3	1
Total	50	45	5
Dry foods:			
Stock	122		122
"Dryco" diet (low fat content)	9		9
Dried biscuit diet	4		4
Total	135		135
Liquid or semi-liquid foods:			
Fresh cows' milk	8		8
Cooked cereal and milk	5		5
Total	13		13

stomach, sometimes more than one, where they apparently lodge, unable to pass the pyloric sphincter.

Although the dry or liquid foods may be left in the cages an equal length of time, there is no tendency for hairs to collect on the surface and the lower fat content lessens the possibility of the hairs sticking together and forming wads in the stomach. It is also possible that a bulky diet would tend to increase elimination of hair except where the results of the high fat content of the paste food predominated.

The constant irritation of the hair balls may cause an inflammation of the stomach mucosa and in several cases microsopic examination has dis-

closed a definitely ulcerated condition. Since the larger balls are usually found in the older rats or in those that have been on the diet for at least three or four months, we have concluded that they must form slowly and therefore do not cause retarded growth or poor physical condition during the first few months, consequently normal growth has been universally observed on these "synthetic" diets. It may be, however, that the constantly increasing irritation of these foreign bodies in the stomach would materially decrease resistance to infection and produce a weakened condition, which might have some bearing on the ability of females to bear and raise litters.

If the preceding hypothesis is correct, a reduction in the fat content of the foods should reduce the number of hair balls, thereby favoring a better physical condition. The results obtained by Evans and Bishop (2) with rats on such diets indicate that the "fat free" foods not only caused no ill effects but were even beneficial in certain cases. In our own experience a modification of the Osborne and Mendel diet made by reducing the fat to such an extent that the food is of a dry, crumbly consistency, lessened the tendency for hairs to form into balls and an apparently better condition resulted in the mother rats on that diet. This experiment is still in progress. Evidence that such a reduction of the fat has no detrimental effect on growth is furnished by Osborne and Mendel themselves (3). We have also noted that the nine rats which were fed on a dry milk diet, containing some fat but crumbly in consistency, were entirely free from hair balls and these have raised several good normal litters. It would obviously be necessary to use alfalfa or a similar source of vitamin A if all fat were omitted, but it seems from our experience that by omitting the lard or crisco, the total of butter-fat or cod-liver oil may still be used without bringing the food to a paste consistency.

In checking up the records of the females in which hair balls were subsequently found, it was noted that there was unusually poor reproduction and if there was more than one litter the number and survival of the later litters was poorer. This would indicate a progressive deterioration possibly related to the formation and irritation of hair balls which might interfere with proper digestion and assimilation of food.

Whether the foregoing hypothesis and conclusions are accepted or not, the fact remains that any animal in such a condition as has repeatedly been found is no fit subject for experimental work which assumes the general well-being of the animal except for the particular deficiency in question. Since this condition might affect control and experimental animals indiscriminately, results would be inconclusive.

SUMMARY

1. Ninety per cent of the rats on paste foods containing 25 to 27 per cent of fat develop hair balls in the stomach.

2. Out of 135 rats on dry foods, *none* developed hair balls.

3. Out of 13 rats on liquid foods, *none* developed hair balls.

4. Formation of hair balls is attributed to high fat content and sticky consistency of the food.

5. Constant irritation by these hair ball often causes inflammation and ulceration of the gastric mucosa.

6. Since these balls form slowly they do not interfere with normal growth but may cause a weakened condition of the adult rat.

7. Reduced fat content of foods has proven beneficial in our own experience and in that of other workers, and such a modification of the Osborne and Mendel diet is suggested.

BIBLIOGRAPHY

(1) Ferry: Journ. Lab. Clin. Med., 1920, v, 735.
(2) Evans and Bishop: Journ. Met. Research, 1923, iii, 233.
(3) Osborne and Mendel: Journ. Biol. Chem., 1920, xlv, 145.

GASTRIC SECRETION AND THE "ALKALINE TIDE" IN URINE

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In 1919 Leathes (1) suggested that the alkaline tide in urine was not due to the secretion of hydrochloric acid by the stomach, and the subsequent reabsorption of the acid from the intestine, but resulted from the change in rate of the respiration incident to the difference between the activity of subjects at night and in the daytime. He described experiments to show that forced respiration increased the alkalinity of the urine, an observation which has been confirmed by the work of others (2), (3), and showed that the alveolar carbon dioxide tension was changed when the subject rose. Other points which he made will be discussed later in this paper. Since the article by Leathes was published, Campbell (4) and Fiske (5) have reported data which tend to show that the alkaline tide occurred regularly after meals, and could probably be attributed in part at least to the secretion of hydrochloric acid by the stomach. In the paper presented below data are discussed which, in the opinion of the authors, tend to support the position taken by the two last-named observers.

In table 1 is given a presentation of results on the reaction of the urine, a preliminary report of which has been given elsewhere (6). Investigations were carried out on 8 cases of achlorhydria (listed in the first part of the table) and on nine patients who showed hydrochloric acid in the gastric contents. Analyses of the gastric juice of all were made by the method described by Rehfuss and his collaborators (7). Urine specimens were collected at intervals of two hours during the day, and the night voidings were combined as a single specimen. The reaction was determined by the method of Marshall (8), but each specimen was collected in a jar under toluol instead of by the technique described by him. All specimens were analyzed as soon as was possible after they were obtained. Gastric analyses and urine studies were not carried out on the same day, but they were made within a short time of each other.

The results show that the alkaline tide was absent in the urine from patients whose gastric juice did not contain hydrochloric acid; such differences as were found between the reactions of different specimens did

not correspond to the appearance of alkaline tides such as are shown in the latter part of the table. The correspondence between the gastric analysis and the alkaline tide conforms to the suggestions in the work of Bennett and Dodds (9) on the relationship between the findings of gastric analysis and the carbon dioxide tension.

The rest of the data presented in this paper was obtained from the study of two normal men (R. S. H., 37 years old, and E. G. A., 25 years old) who were doing light laboratory work during the period of study.

TABLE 1

Cases of achlorhydria

TIME	CASE NUMBER								
	A1	A2	A3	A4	A5	A6	A7	A8	Average
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
7–9	5.8	5.8	6.6	5.1	5.15	6.3	5.15	5.3	5.7
9–11	5.6	5.8	6.1	4.9	5.3*	5.25	5.15	5.35	5.4
11–1	5.6	5.3	6.4	5.2	5.3*	4.9	5.05	5.2	5.4
1–3	5.5	6.2	5.9	5.1	5.45	4.8	5.25	5.4	5.5
3–5	5.65	5.7	6.1	5.25	5.4	4.9	5.2	5.45	5.5
5–7	5.5	5.3	7.2	5.4	5.7	4.9	5.4	5.3	5.6
7–7	5.7	5.6	6.4	5.2	5.3	5.0	5.4	5.7	5.5

Hydrochloric acid present

TIME	CASE NUMBER									
	B1	B2	B3	B4	B5	B6	B7	B8	B9	Average
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
7–9	5.5	5.5	6.4	5.65	5.1	6.15	6.4	4.8	5.3	5.6
9–11	7.1	5.7	6.9	5.8	5.4	6.9	6.0	5.0	6.4	6.1
11–1	5.3	5.9	6.9	5.55	6.6	7.7	5.4	7.0	6.6	6.3
1–3	5.85	5.3	6.7	5.0	5.0	6.0	5.0	5.0	5.3	5.5
3–5	6.7	6.1	7.7	6.0	5.1	5.9	5.0	6.0	5.3	6.0
5–7	5.7	5.45	8.0	5.3	5.1	5.6	5.05	5.4	5.3	5.7
7–7	5.5	5.6	6.4	4.9	5.4	5.9	5.1	4.9	5.4	5.5

* Only one specimen voided in these two periods.

Hydrochloric acid had been demonstrated by the method described above in the gastric secretion of both subjects. In these experiments the urine was collected and analyzed by the method of Marshall (8). Various breakfasts were used in these tests, and the urine was collected at hourly intervals throughout the morning. The breakfasts used are described in table 2.

In table 1 three patients (B5, B7, B9) whose gastric juice contained free hydrochloric acid failed to show an alkaline tide in the afternoon. Leathes (1) reported similar findings, and was inclined to infer from them

that no alkaline tide occurred when general equilibrium had been established and the rate of carbon dioxide elimination had become adjusted to the conditions pertaining during the daytime. It has been suggested (10) that such findings may be due to the excretion of acid products from the first meal eaten, for such products might prevent the development of the second tide. Both Campbell (4) and Fiske (5) have shown that tides frequently, if not regularly, develop after the second meal of the day. Table 3 presents data similar to the more extensive data of these authors. After a comparatively simple breakfast and a fairly heavy dinner, the second tide was present.

TABLE 2

Breakfasts

NAME	DESIGNATION	FOODSTUFFS	FOODS	
				grams
Standard	S	60 gm. toast, 13 gm. butter, 50 gm. egg, 200 cc. milk, 250 cc. water	Carbohydrate Protein Fat	42 19 26
High carbohydrate 1	C1	88 gm. pitted dates, 5 gm. sugar, 12 gm. shredded wheat, 468 cc. water	Carbohydrate Protein Fat	82 3 3
High carbohydrate 2	C2	12 gm. shredded wheat, 60 gm. toast, 350 cc. water	Carbohydrate Protein Fat	65 9 1.5
High protein	P	85 gm. egg, 30 gm. cheese, 160 gm. steak, 300 cc. water	Carbohydrate Protein Fat	2 52 42
Water	W	500 cc. water	None	
Fasting	F	None	None	

Leathes reported that the morning tide persisted if no meal was taken, and Campbell that the tide was lacking in 80 per cent of his cases in which breakfast had been omitted. In table 4 data from two experiments in which no food was taken and from one in which water only was taken at the usual breakfast time are presented. Determinations after a simple standard breakfast were made at about the same time, and the results of these experiments are given to facilitate comparison. The alkaline tide either was absent or was markedly diminished when breakfast was omitted.

In table 5 data are given to show the effect of different types of breakfast upon the alkaline tide. A tide was present in all cases. It was most

TABLE 3

Subject R. S. H. Standard breakfast (S) at 8:30, dinner ad lib. at 1:15

	TIME										
	7–8	8–9	9–10	10–11	11–12	12–1	1–2	2–3	3–4	4–5	5–6
Reaction, pH.....	5.5	6.2	7.4	7.4	7.0	6.3	6.0	7.0	7.4	7.1	6.8

TABLE 4

Effect of lack of nourishment on the alkaline tide

Part 1. Subject R. S. H.—fasting

JULY 5, 1923, STANDARD BREAKFAST (S)			JULY 3, 1923, NO BREAKFAST (F)		
Time	Volume	Reaction	Time	Volume	Reaction
a.m.	*cc.*	*pH*	*a.m.*	*cc.*	*pH*
7–8	28.0	5.5	7–8	34.0	5.4
8–9	26.5	5.9	8–9	17.5	5.8
9–10	24.0	6.9	9–10	11.5	5.9
10–11	71.0	6.9	10–11	15.0	5.6
11–12	59.0	6.7	11–12	11.5	5.6
12–1	61.0	6.4	12–1	21.0	5.6

Part 2. Subject E. G. A.—fasting

JULY 6, 1923, STANDARD BREAKFAST (S)			JULY 3, 1923, NO BREAKFAST (F)		
Time	Volume	Reaction	Time	Volume	Reaction
a.m.	*cc.*	*pH*	*a.m.*	*cc.*	*pH*
7:20– 8:20	27.0	5.4	8:15– 9:15	22.0	5.3
8:20– 9:20	27.0	5.6	9:15–10:15	18.0	5.3
9·20–10·20	34.0	6.2	10·15–11:15	24.5	5.3
10:20–11:20	48.0	6.0	11:15–12:15	19.5	5.2
11:20–12:20	46.0	5.8	12:15– 1:15	22.0	5.1
12:20– 1:20	41.0	5.8			

Part 3. Subject E. G. A.—water only

JULY 12, 1923, STANDARD BREAKFAST (S)			JULY 16, 1923, WATER (W)		
Time	Volume	Reaction	Time	Volume	Reaction
a.m.	*cc.*	*pH*	*a.m.*	*cc.*	*pH*
7– 8:12	44.0	5.7	7:30– 8:30	32.0	5.2
8:12– 9:12	21.5	5.8	8:30– 9:30	26.5	5.3
9:12–10:12	32.0	6.3	9:30–10:30	29.5	5.3
10:12–11:12	45.0	6.7	10:30–11:30	29.0	5.2
11:12–12:12	57.0	6.6	11:30–12:30	23.5	5.1
12:12– 1:12	45.5	5.7	12:30– 1:30	29.5	5.1

marked when the breakfast was high in protein, and was least pronounced when that meal was highest in carbohydrate. These results agree with such data as are available upon the effect of different foods upon the secretion of acid by the stomach, for beef which was present in the high protein breakfast causes the production of a higher concentration of acid

TABLE 5

Different breakfasts fed to subject E. G. A.

TIME	JULY 10, 1923, S MEAL		JULY 18, 1923, C_2 MEAL		JULY 20, 1923, P MEAL		S-AVERAGE THREE EXPERIMENTS
	cc.	*pH*	*cc.*	*pH*	*cc.*	*pH*	*pH*
1 hr. before meal	32.5	6.6	5.0	5.4	12.5*	5.2	5.9
1 hr. at meal time	25.0	6.9	19.5	5.3	24.0	5.4	5.4
1 hr. after meal	31.5	6.9	25.5	5.5	46.0	6.8	6.8
2 hrs. after meal	43.0	7.2	20.0	5.7	54.0	7.2	6.6
3 hrs. after meal	39.0	6.5	35.5	5.6	37.0	6.3	6.3
4 hrs. after meal	36.5	6.0	30.0	5.3	31.0	5.4	5.8

* This specimen collected for only half an hour.

TABLE 6

Alkaline tide and gastric analysis on diet high in carbohydrate (C1)

FIRST EXPERIMENT AUGUST 23, 1923						SECOND EXPERIMENT SEPTEMBER 6, 1923					
Gastric			Urine			Gastric			Urine		
Time	cc. 0.1 N in 100 cc. HCl	cc. 0.1 N in 100 cc. Total	Time	Volume	Reaction	Time	cc. 0.1 N in 100 cc. HCl	cc. 0.1 N in 100 cc. Total	Time	Volume	Reaction
	cc.	*cc.*		*cc.*	*pH*		*cc.*	*cc.*		*cc.*	*pH*
8:40	0	23	7:30– 8:30	26	5.4				7:32– 8:37	35	5.6
9:10	0	88	8:30– 9:30	39	5.4				8:37– 9:37	47	5.6
9:40	0	110	9:30–10:30	57	5.3				9:37–10:37	76	6.6
10:10	0	71	10:30–11:30	45	5.0				10:37–11:37	34	6.7
10:40	18	78	11:30–12:30	39	5.2	10:45*	35	47	11:37–12:37	26	5.7
11:40	0	12									
12:40	0	7									

Breakfast in both experiments eaten at 8:45.

* Only one gastric specimen obtained by passing tube at this time.

than do eggs, which in turn cause a more strongly acid secretion than do meals consisting of cereals and bread (11).

It would be desirable to carry out determinations upon the production of hydrochloric acid in the stomach and variations in the alkaline tide simultaneously. We feel that such experiments are open to objections of two kinds: first, the effect of the repeated withdrawals of samples of

the gastric contents upon the excretion of acid by the kidneys is uncertain; secondly, the presence in the gastric contents of large amounts of buffer material from the food may affect the interpretation of the values obtained by the titration of the gastric contents. In table 6 the results of two experiments are given which illustrate these difficulties, but which are nevertheless of interest in the present study. In the first experiment the tube was swallowed, a breakfast high in carbohydrate eaten, and determinations upon the gastric contents and the acidity of the urine carried on simultaneously. No alkaline tide was shown in the urine, and hydrochloric acid was found in only one of the gastric specimens, and in this specimen the amount was small. Values obtained by titration to the phenolphthalein endpoint were very high, however, as contrasted with the values obtained on the same subject in other experiments, and it seems to be doubtful whether much emphasis can be placed upon the apparent lack of free hydrochloric acid observed. In an experiment done about two weeks later in which the same breakfast was eaten, but in which the tube was not introduced at the beginning of the experiment, a definite alkaline tide developed. Half way through the experiment a gastric tube was swallowed, and a single sample of gastric juice containing a fair amount of hydrochloric acid obtained. If this experiment could be accepted it would furnish strong evidence in support of the theory that the alkaline tide is caused by the secretion of hydrochloric acid by the stomach, but it seems to us that the results can be accepted only with a considerable amount of reservation.

The evidence from experiments which show an absence of the alkaline tide in cases with failure of the stomach to secrete hydrochloric acid; from the presence of two alkaline tides in most of the subjects investigated; from the absence of the morning tide when no nourishment was taken into the stomach; from the resemblance between the intensity of the tides following different types of breakfast and the reported effect of similar diets upon gastric secretion,—seems to indicate that the secretion of hydrochloric acid by the stomach is an important, if not the most important factor in producing these variations in the acidity of the urine.

BIBLIOGRAPHY

(1) Leathes: Brit. Med. Journ., 1919, ii, 165.
(2) Collip and Backus: This Journal, 1920, li, 568.
(3) Grant and Goldman: This Journal, 1920, lii, 209.
(4) Campbell: Biochem. Journ., 1920, xiv, 603.
(5) Fiske: Journ. Biol. Chem., 1921, xlix, 163.
(6) Hubbard and Munford: Proc. Soc. Exper. Biol. Med., 1922, xix, 429.
(7) Rehfuss, Bergeim and Hawk: Journ. Amer. Med. Assoc., 1914, lxiii, 909.
(8) Marshall: Journ. Biol. Chem., 1922, li, 3.
(9) Bennett and Dodds: Brit. Journ. Exper. Pathol., 1921, ii, 58.
(10) Hubbard and Munford: Journ. Amer. Med. Assoc., 1923, lxxx, 304.
(11) Rehfuss and Hawk: Journ. Amer. Med. Assoc., 1920, lxxv, 449.

AQUEOUS EXTRACTS OF PANCREAS

IV. The Yield of Crude Insulin by Perfusion, Percolation and Simple Extraction

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Before proceeding to a detailed description of the method of purification of insulin which has given the best results in this laboratory, it seemed advisable to restudy the several steps with a view of increasing the total yield. The first of these steps is the removal of crude insulin from the pancreas of the pig or ox. No other pancreas is available in such quantity or is likely to become so available in the near future.

Three distinct methods of using water extraction have been employed by us from time to time, each presenting several points of superiority over the other two. Assuming that the crude product would be equally amenable to further purification the determining factors in the choice of a method for large scale production obviously would be: first of all, total yield, and second, ease or cost of manipulation. The three methods are perfusion, percolation and simple extraction.

I. The perfusion method. But few investigators appear to have undertaken direct perfusion of the pancreas. The most complete study of this type of perfusion was reported by Dr. A. H. Clark in 1916 (1). His work seems to have stimulated all work of this kind since that date. While our earlier work on pancreatic perfusion was under way a paper by Landes, Garrison and Moorhead appeared (June, 1922) (2). They state that they were able to find in the literature but two other references to direct perfusion of the pancreas besides Clark's—one by Hustin, relating to a study of the external secretion of the pancreas, and another by de Meyer relating to glycogen storage by a liver perfused with a solution previously used in perfusing a pancreas. Very recently (July, 1923) a second perfusion paper from the University of Chicago laboratory was published by McCarthy and Olmstead (3). This work was begun in 1920. Neither of these groups of investigators had any success with pancreatic perfusion—in fact, they do not appear even to have confirmed Clark's observations.

Ever since October, 1921, when we began the present series of experiments upon methods of obtaining the anti-diabetic substance from the

pancreas, we have been developing the perfusion method (4), (5). We first employed it in the hope of eliminating toxic properties which had been encountered in extracts made with dilute hydrochloric acid. Following the successful demonstration of the glucopyretic substance we continued the method as a check upon other methods which were developed from time to time. Our earliest experiences pointed to the apparent superiority of the perfusion method over others, from the standpoint of the elimination of toxic effects upon experimental animals, and from the standpoint of yield of anti-diabetic substance per gram of pancreas used. The relative absence of large amounts of protein and other troublesome products in perfusates made it possible to employ them in experiments upon depancreatized cats and dogs without further treatment beyond the adjustment of the reaction. In short, it seemed that by this method we were obtaining a product which was practically free from the external secretion of the pancreas and extraneous tissue extracts—one which represented more nearly the true internal secretion of the pancreas. This meant not only that a more satisfactory product was obtained in this manner but also that there was a great saving of time and labor in its preparation.

Our first perfusions were carried out under strictly aseptic conditions and were rather close repetitions of those experiments of Clark in which he demonstrated the utilization of glucose by the isolated heart when perfused with Locke's solution which had previously been circulated artificially through the pancreas of the animal from which the heart was isolated. Our object differed fundamentally from his in that we sought to show whether perfusates obtained in this manner would affect the respiratory exchange of depancreatized animals. The successful demonstation of a positive effect upon the respiratory exchange (glucopyretic effect) has been reported in two previous papers (4), (5).

Our first experiments were concerned with the perfusion of the organs removed during pancreatectomy and the subsequent use of these perfusates upon the same animals or upon others of the same species—e.g., a perfusate of cat's pancreas was used to control the diabetic condition of cats. Later, perfusates of dog's pancreas were used to control the diabetes of cats, and finally the attempt was made to utilize slaughter house material in the form of pig's pancreas. This obviously would make it possible to produce the anti-diabetic substance in greater amount. Some of our first experiments in this direction were highly successful (5). This transition was highly desirable from the standpoint of permitting an unlimited supply of material, but in itself it introduced some new problems. In the first place, it became clear that asepsis could no longer be attained because, by the nature of things, we were dealing with contaminated organs. Again, it would no longer be possible to deal with organs at exactly body

temperature. In our earlier experiments it was easy enough to connect the extirpated pancreas with the perfusion apparatus at once so that the organ could be kept at body temperature until the perfusion had been completed. In dealing with slaughter house material we did succeed in maintaining the organs at body temperature by transporting them in a thermos jar, but frequently considerable time elapsed before all cut vessels could be ligated and the perfusion begun. Our experiments automatically eliminated the temperature factor as of paramount importance for we soon obtained successful perfusates from organs which, at the time the perfusion was begun, had been removed from the animals for two hours and were distinctly cold to the touch. By this time also the laboratory staff had succeeded in developing methods by which potent material removed from the pancreas could be precipitated, separated from protein, and concentrated. These methods solved our problem of removing bacterial contamination and made it possible to administer doses of a few cubic centimeters instead of the large doses of 25 to 100 cc. which were needed when we were using unrefined and unconcentrated products.

Next followed a series of 29 perfusions in which various modifications were made in the conditions under which the perfusions were done. All variable factors were recorded with the object of making an analytical study in order to determine the essential factors concerned in successful extraction by perfusion. Modifications were made in each of the following: pressure at which the perfusing fluid was delivered to the pancreas; composition of the perfusing fluid; temperature of the perfusing fluid and of the pancreas during perfusion; rate of perfusion; volume of perfusate; and time of perfusion. We have selected for analysis four perfusates (nos. 11, 26, 34 and 38) from this series. These four were equally potent (as shown by animal tests) and include all of the important variables involved in the method.

The success of these perfusions from the mechanical standpoint was judged from the results of injection of the blood vessels with a colored injection fluid. In all of these cases it was shown that the perfusing fluid had reached all parts of the organs.

The time of perfusion does not seem to be a critical factor. In the four cases cited the time varied from one to two hours. The time and rate determine the total volume of solution passing through the pancreas and we have found that apparently one hour is efficient when fluid is being continuously supplied under a pressure of 120 mm. of Hg or higher.

For perfusing fluids we employed on different occasions: Ringer's solution containing HCl varying from 0.2 per cent to 0.7 per cent, oxygenated and not oxygenated; distilled water alone and with bile; ethyl alcohol 5 per cent and 50 per cent with 0.2 per cent HCl; 5 per cent and 10 per cent sulphuric ether with 0.2 per cent HCl; 0.2 per cent HCl with 5 per cent

sulphuric ether and 5 per cent ethyl alcohol; 0.1 per cent HCl alone; and 0.2 per cent HCl alone. Although Ringer's solution slightly acidulated proved to be a good solvent for the anti-diabetic substance it was desirable to eliminate Ringer's solution, if possible, because of the excessive salt content brought about by concentration of the perfusates. This, in turn, necessitated dialysis, and dialysis for a sufficient time to remove the salts appeared to carry away potency (6). For example, perfusate 34 was divided into three portions after the perfusion was completed. The first portion was dialyzed over night in an open tray in still water; a second portion was dialyzed over night in closed sacs in running water; and a third portion was not dialyzed until near the end of purification when it was dialyzed in a closed sac for four hours in running water. The last portion was the most potent of the three (small doses produced convulsions in four different rabbits) and the first had nearly lost its potency. Our early perfusion experiments (5) established beyond a doubt the superiority of the acidulated solutions for perfusion. Preliminary trials with distilled water and 0.1 per cent HCl having proved unsuccessful, we continued to use the strength of acid employed in the first experiments, namely, 0.2 per cent. Indeed, this strength has seemed ideal for perfusion. With higher percentages of acid considerable physical change was produced in the organs and there appeared to be a loss of permeability. Ether was employed with the idea of modifying the surface tension conditions of the cells of the islet tissue but no superior results were obtained by this method. We were gradually able to eliminate other substances and to conclude that acidulated water is an ideal solvent for the anti-diabetic substance as it comes from the cells. The strength of acid found necessary to overcome interfering substances likewise proved to be a good bactericidal agent.

At first all perfusions were done in an incubator strictly at body temperature; later the perfusing fluid was kept at body temperature and the pancreas at room temperature. During perfusion the capsule of the pancreas becomes widely separated from it by the accumulation of a deep layer of fluid. By thrusting a clinical thermometer through this layer and into the interior of the pancreas we have repeatedly found that the pancreatic tissue has the same temperature as the perfusing fluid so that nothing is lost by perfusing in the room. Swelling of the pancreas is facilitated by a warm solution and this in turn leads to better penetration of the capillaries by the perfusing fluid. There is practically no drying of the surface of the organs during perfusion owing to the fact that it is constantly bathed by the escaping fluid.

In the first three of the group of most successful perfusions a pump was used, the pulse pressure being 32, 104 and 205 mm. Hg respectively. These three perfusates were about equally potent. As compared with

no. 11, perfusate 26 was made with a pulse pressure about three times as high and no. 34 was made with a pulse pressure about six times as high. The pulse pressures of successful perfusions have varied from 20 mm. Hg to as high as 205 mm. Hg. Perfusate 38 was the first one done under continuous gravity pressure. This preparation was successful and the penetration of the pancreas was as good as that secured in the previous experiments with intermittent pressure. Since that time gravity perfusion has been adopted as the method of choice.

There is probably an optimum relation between the number of cubic centimeters of fluid and the number of grams of pancreas perfused. For the purpose of determining this we have calculated the number of cubic centimeters of perfusate per gram of perfused pancreas. This is a product of three factors: weight of perfused pancreas, volume of fluid delivered in unit time, and the time of perfusion. In the first three of the perfusions the number of cubic centimeters of perfusate per gram is 54 for no. 11; 227 for no. 26; and 169 for no. 34. Here is a variation of four- and three-fold respectively. Judging from the results obtained in perfusate 11, 54 cc. of solution passing through each gram of pancreas washed out as much of the anti-diabetic substance as did the larger quantities. The figure for no. 38 is not known since at the time of the first gravity perfusion we had no method of calculating the volume of fluid delivered to the pancreas.

II. Object of the present series of experiments. As a result of the recent review (7),(8) of the results of the various methods employed at our laboratory for the preparation of insulin we were struck with the apparently greater yield by the perfusion method. In our most successful experiments (perfusates 26, 34, 38) we were able to obtain approximately three times as much anti-diabetic substance per kilo of pancreas used, as we had been able to obtain in our most successful simple extractions. There was, however, one factor which was essentially different in the two processes—that is, the species of pancreas used. For most of the simple extractions ox pancreas had been used because of the fact that this type of pancreas has greater weight per organ, and also because it contains relatively little fat (and this is easily removed). For perfusion we had employed chiefly pig pancreas because of the smaller size of the organs and the consequent greater ease in handling in the apparatus which we have used.

The present series of experiments was designed to attempt to settle the question of the apparent superiority of the perfusion method over other extraction methods for obtaining the anti-diabetic substance, by utilizing the pancreas of the same species. Parallel lots of pig pancreas have been carried along by the most successful simple extraction methods, by percolation, and by the simplest efficacious perfusion method. After the anti-

diabetic-substance has been obtained in solution by the different methods, the lots of solution have been purified and tested in the same manner as nearly as possible. As will be noted below, it has proved impossible to remove the proteins from the several kinds of fluids by identical treatments;[1] but the difference consisted only in a variable pH. All other facts were comparable.

In this study we have used the terms "perfusates," "percolates," and "simple extracts" to designate different methods of extracting insulin from the pancreas. We have never considered that the pancreas survives (it obviously does not) during the process of perfusion by the methods which we have employed in this study, but rather that perfusion is a modified extraction method possessing the following advantages: relative absence of cell injury; intimate contact of the perfusing fluid with the islet tissue; and a filtration effect from the capillary bed. The elaborate experiments of E. R. McCarthy and H. C. Olmstead (3) at the University of Chicago show that during perfusion the pancreas shows no evidence of active survival. These investigators sought, with great care, to reproduce physiological conditions in all possible details (even using defibrinated blood for the perfusing fluid) and showed that there is no evidence of the production of the external secretion of the pancreas during perfusion. The production of the external secretion of the pancreas was taken as the criterion of survival of the organ.

Likewise percolation is clearly an extraction method, but it, too, possesses certain distinguishing features.

We have grouped methods other than the special ones embraced by perfusion and percolation under the title "simple extracts."

III. LARGE SCALE PERFUSION METHOD. *1. Apparatus.* The apparatus used for the present series of experiments has resulted from many modifications of that originally used. Since the perfusion fluid must contain acid it is obvious that only glass or acid-resisting enamel can be used in construction because of the fact that dilute acids act upon nearly all metals or alloys. The largest glass pump which we used during our earlier experiments consisted of a 50 cc. Luer syringe driven by a motor with reducing gears and eccentric. Owing to the slow filling we were able to secure an output of only 350 cc. per minute—an amount which, in the light of later experiments, we have found to be sufficient for but a single pig pancreas. This limitation of output caused a consequent limitation of the amount of pancreas which could be perfused. With the demonstration (perfusate 38) of the fact that gravity perfusion under constant pressure could be used successfully we turned our attention to designing apparatus for this purpose.

[1] A full description of the methods of handling these fluid extracts of pancreas so as to obtain the strongest insulin reaction will be found in the next paper of this series.

The essential features of the present apparatus are: first, elimination of all contact of the perfusing fluid with metal of any kind; second, the elimination of delicate glass valves actuated by pressure changes in the system; third, the supplying of unlimited quantities of perfusing fluid to the pancreases under constant pressure; fourth, the use of compressed air to move the fluid from a low level to a high level. The parts of the apparatus are shown in figure 1.

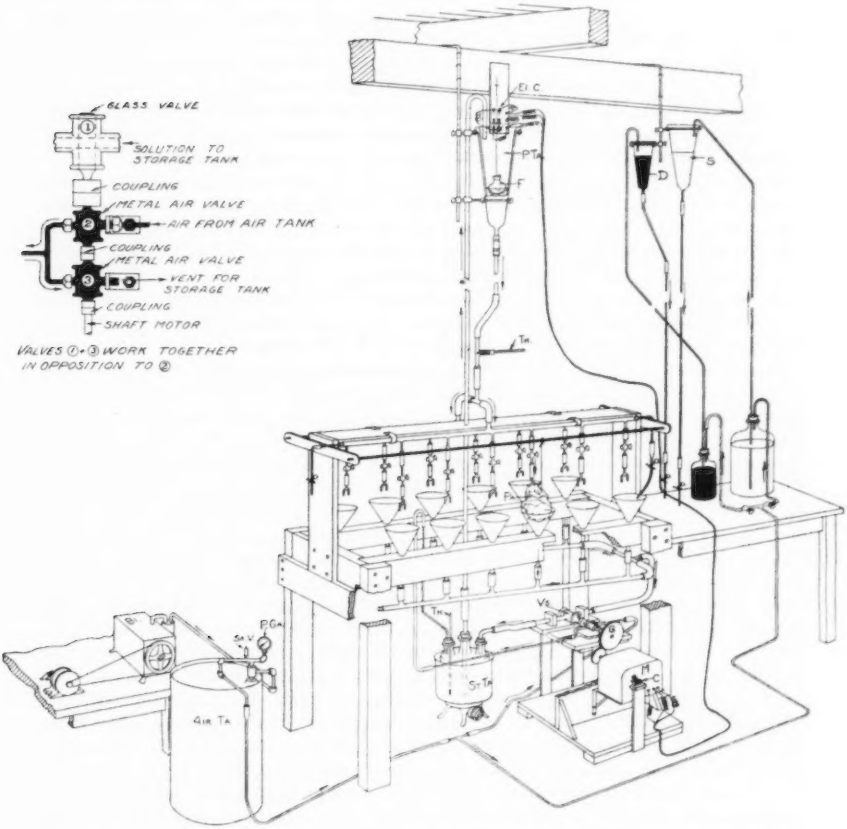

Fig. 1. Apparatus used for removal of insulin from the pancreas by perfusion. Twelve organs can be perfused at one time. Description in text.

The apparatus consists of: *a*, a glass tank of small capacity for supplying fluid under gravity pressure (this is known as the "pressure tank"); *b*, arrangements for collecting the fluid which drains from the pancreases during perfusion; *c*, a storage tank where the main volume of fluid can be heated and from which it can be supplied to the pressure tank by compressed air; *d*, an electric motor turning a shaft which controls the position of the valves supplying compressed air and the fluid returning

from the pancreas after passing through them; and *e*, apparatus for supplying compressed air.

a. Pressure tank. A tank, *P. Ta.*, of but small capacity is needed for this purpose. Only enough fluid is permanently held in this tank to supply the organs during the interval while the storage tank below is supplying the pressure tank with fresh fluid. A rapid interchange of fluid between these two tanks is desirable in order to prevent excessive loss of heat from the perfusing fluid. For our apparatus (which was designed to take care of twelve pig pancreases) a glass percolator jar of two liters capacity is sufficient. This is placed at a height of 250 cm. above the level at which the pancreases are perfused and supplies a pressure of 200 mm. Hg at the pancreases. It is obvious that this tank can be increased to any size which may be needed.

A glass float, *F.*, rising and falling with changes in the fluid level in this tank controls the operation of the compressed air valve connected with the storage tank: when the fluid level falls to a certain point contact is made, by means of a set of mercury cups, *El. C.* on the float, with the motor, *M.*, which turns the compressed air valve admitting air to the storage tank; after the fluid level has risen to a certain point contact is again made, by means of a second set of mercury cups, with the motor, which revolves the valve shaft, *Vs.*, a quarter turn, shutting off the compressed air. The height of the float is adjustable so that the volume of fluid residual in the pressure tank can be controlled. The distance through which the float moves can be controlled. Once these adjustments have been made for a given set of conditions no further attention needs to be given to the float. Since the operation of the valves is directly controlled by this float it must be rigidly and carefully made. A small glass flask, *F.*, 7 cm. in diameter, with a neck 4 cm. high and 3.5 cm. in diameter, is used for the ball of the float. This rides on the top of the fluid so that only the glass is in contact with the perfusing fluid. Attached to the top of the flask are three rods of brass tubing 2.5 mm. in diameter and 28 cm. long, running through guides. Attached to the rods is a triangular piece of hard rubber carrying half of the electrical contacts. These contacts consist of small iron cups filled with mercury into which pieces of German silver (or better platinum) wire dip.

Connected to the bottom of the pressure tank is a straight glass tube 15 mm. internal diameter leading to a distributing tube of similar size placed about a foot above the level at which the perfusions are done. The distributing tube occupies an horizontal position and is at right angles to the supply tube. Connected to the lower side of the distributing tube are six outlets 6 mm. internal diameter. By means of a short piece of rubber tubing a glass stopcock is attached to each of the six outlets. A short length of rubber tubing is used to connect the stopcocks with the cannulas placed in the pancreases. In each end of the distributing tube is a rubber stopper containing a short length of glass tubing to which a short piece of rubber tubing is attached. The rubber tubing is held closed with a stiff pinch clamp. The object of placing these two tubes in the ends of the distributing tube is to permit one to release the air bubbles which accumulate there when the first portion of perfusing fluid is allowed to flow down from the pressure tank. Once the system is completely filled with fluid no further attention is paid to it for no more air is admitted. A thermometer, *Th.*, is inserted into the tube leading down from the pressure tank, through a side tube, in order to show the temperature of the perfusing fluid as it is supplied to the pancreases.

b. Drainage arrangements. The fluid draining from the pancreases is allowed to drip into funnels. Separate funnels are placed for the individual pancreases. These are connected into a common large tube (15 mm. internal diameter) by means of side tubes varying in length, by half inches, from 2½ to 5 inches in order to give sufficient inclination. Fluid drains through this tube into the storage tank through a large

glass stopcock (10 mm. bore through the stopper). An enameled trough as wide as the top of the funnels and with sufficient inclination would serve as well.

c. Storage tank. The storage tank, *St. Ta.*, can be of any desired capacity but should be of glass. We have found a two-liter Woulff bottle of sufficient size for perfusing six pig pancreases. This bottle is located below the level of the drainage tube from the pancreases and would best be 2 or 3 feet below this level in order to secure a rapid flow of fluid, under gravity pressure, from the drainage chamber. The tank is placed in a larger vessel containing enough water to nearly cover the storage tank. This water chamber rests upon a small electric hot plate which supplies the heat necessary for raising the temperature of the perfusing fluid to the proper point.

Three tubulures in the top of the bottle are used for the following purposes: into the first is placed a rubber stopper which just admits a glass tube with internal diameter of 10 mm. (this connects with the drainage chamber through the large glass stopcock); into the middle tubulure is placed a large glass tube 15 mm. internal diameter, extending to the bottom of the tank (this extends up to the pressure tank); into the third tubulure is placed a two hole rubber stopper containing a thermometer, *Th.*, extending down to the middle of the chamber and a glass tube of about 6 mm. internal diameter just reaching through the stopper. This glass tube extends upward to a height just in excess of that of the drainage funnels where it is joined to a copper tube of the same size. This arrangement supplies compressed air to the storage tank, at the proper times. It is important that the glass portion of this line extend to the height indicated because, the apparatus being designed to hold all of the fluid used for perfusion in the drainage vessels and the storage tank, fluid backing up in the funnels would also back up into this glass tube and in the absence of an air trap would pass out through the vent (see below). When the storage chamber is full the fluid will rise in this tube. All three rubber stoppers in the Woulff bottle, or storage tank, should be wired in order to withstand the pressure of the compressed air. A thermometer (not shown) is inserted into the tube leading from the bottom of the storage tank to the pressure tank, a short distance above the storage tank, through a side tube. This indicates the temperature of the fluid as it leaves the storage tank. By noting the temperature of the fluid as it leaves the storage tank and comparing it with the temperature of the fluid entering the distributing tube, it is possible to determine the heat lost by the fluid during its circulation. The fluid is usually circulated through the apparatus for 10 to 15 minutes before perfusion is begun in order to heat the glass. After this has been done it has been found that heat loss seldom amounts to more than one to two degrees Centigrade.

d. Electric motor controlling valve shaft. Three stopcocks are used to control the flow of fluid and the flow of compressed air. These are mounted in a straight line on the same shaft, *Vs.*, which is controlled by the motor, *M*. The first stopcock is a large glass one through which the perfusing fluid flows in its course from the drainage tube to the storage tank. The other two stopcocks are small and made of bronze. The stopcocks are set so that the glass one and one of the bronze cocks work together and in opposition to the other bronze cock. The reason for this is clear—when air is admitted into the storage tank to raise the fluid by pressure, it must not be allowed to escape through the vent valve or through the drainage valve. The operation of the stopcocks is simple and dependable because the stopcocks are permanently fastened to the same shaft: when the first two are closed the third is open and vice versa. The stopcock controlling the vent from the storage tank is set so that it opens slightly before the glass one opens. An advance of 5° is sufficient to open this valve. The reason for adjusting the valves in this manner is to allow the excess air pressure in the storage tank to escape through the vent so that it will not back up through the drainage valve and meet the fluid which is coming from the

drainage chamber (funnels). A special detail drawing illustrating the action of the three valves mounted on the valve shaft is shown in the insert of figure 1.

The motor which turns the shaft to which the stopcocks are fastened is designed to make a quarter revolution each time that a contact is alternately established by the rise and fall of the float in the pressure tank. For our purpose we found that the electric regulator manufactured by the Minneapolis Heat Regulating Company serves admirably. This small electric motor has its speed reduced, by means of proper reducing gears, to about six revolutions per minute. It is released by two contacts acting alternately. As it is regularly supplied for heat regulating purposes the motor causes a half revolution of the shaft, each time the motor is alternately released. By using two-to-one gears, *G.*, to connect the valve shaft to the motor shaft, a quarter revolution of the stopcocks is produced at each release of the motor. A small transformer mounted on the motor case (below) supplies a current of low voltage and low wattage for the float contacts, thus eliminating the use of batteries of any type. This current of low voltage and low wattage is desirable for the float contacts in that it reduces sparking (and consequently oxidation) at the contacts. The total time required for a complete operation of the apparatus—i.e., shutting off the storage tank, filling the pressure tank, and reëstablishing connection between the storage tank and the drainage apparatus and shutting off the compressed air—is less than ten seconds. The actual time required to fill the pressure tank under the conditions under which we operate (using 12 pounds of compressed air) is scarcely two seconds, the rest of the time being taken up by the motor and gears.

In order to be able to calculate the volume of fluid supplied to the pressure tank we have connected a Veeder Revolution Counter, *C.*, to the motor shaft. This counter thus indicates a complete operation of closing drainage and vent valves, admitting compressed air, and reëstablishing the original relations between the various tanks. By calibrating the float for the complete operation we can determine the amount of fluid supplied to the pressure tank during the entire perfusion period by multiplying the unit volume represented, by the number of revolutions in the perfusion period.

It is by no means necessary to use the float and motor arrangement to control the valve shaft. We have used this automatic type of control because of the fact that we have been working on a small scale. In working on a large scale it is possible to use jacketed tanks for the pressure and storage tanks (thus eliminating heat loss through transfer of fluid) and, by having these of sufficiently large size so that the frequency of transfer of fluid is reduced, the valve shaft can be operated by hand.

e. Compressed air supply. Any arrangement for supplying compressed air under low pressure can be employed. We use a rotary compressor (Crowell blower), *Bl.*, which is connected with a galvanized iron tank, *Air Ta.*, of 36,000 cc. capacity. The tank is provided with a gauge, *P. Ga.*, to indicate the pressure contained, and an adjustable safety valve, *Sa. V.* The blower supplies a maximum pressure of 15 pounds per square inch and we employ a pressure of 12.5 pounds. Seven pounds pressure is sufficient to raise the fluid slowly to the pressure tank but the operation is completed in about two seconds with the higher pressure. The float is adjusted to deliver about 450 cc. of fluid at each filling. It is obvious that increasing the air pressure will diminish the time required to raise the fluid from the storage tank to the pressure tank above. With the large sized delivery tube used between the storage and pressure tanks (15 mm. internal diameter) a huge stream is quickly sent into the pressure tank.

f. Cleaning the apparatus. We have found that a solution of 5 per cent sulphuric ether in 0.5 per cent HCl is efficient for cleaning the apparatus after use. The chief

substance to be removed is the fat which accumulates on the inside of the glass tubes and this is readily removed by the ether and acid solution. After cleaning, the apparatus is kept filled with 0.5 per cent HCl until the next time of operation.

2. Preparation of the pancreas for perfusion. We have used material removed from the animals by men unfamiliar with the anatomical relations of the pancreas beyond its regional position. They take care merely to remove the pancreas in toto as far as possible, but because of the extreme vascularity of the pancreas, they cannot remove the organ without cutting a number of vessels. It is equally clear that successful perfusion cannot be accomplished unless the vascular system of the pancreas is practically intact. Only by forcing fluid under pressure into the capillaries can we hope to perfuse the island tissue.

The first step in the preparation of the pancreas for perfusion is the ligation of all severed arteries (venous channels must not be included within the ligatures). In the pig two large branches from the main artery supply the entire pancreas—one division supplies the head of the organ and another division supplies the body and tail. In removing the pancreas these two branches are almost invariably separated from the main trunk so that we have found it convenient to use a Y-shaped cannula which is securely tied into the two divisions of the main artery. After this has been done the cannula is connected to a glass tube leading from a jar of 0.6 per cent NaCl (*S*, in fig. 1) suspended at a height of 250 cm. to supply fluid at the pressure used for perfusion. The saline is kept at room temperature rather than at body temperature because of the greater possibility of losing some of the insulin if the fluid is warm. The saline under pressure spurts out from the ends of cut arteries; these are seized with hemostats and securely tied with heavy thread. After the vessels have all been secured the pancreas is removed from the saline pressure tank. Without any special preparation beyond the ability to recognize the main vessels, one can easily prepare an organ in five minutes. Frequently we have found that only two ligatures have been needed—a fact which leads us to believe that with a little care in removing the organs very little ligation would be required. The distribution of blood vessels in the pig differs markedly from the distribution in the dog in that in the latter there is a very close anastomosis between the pancreatic and duodenal vessels.

After the ligation of vessels the pancreas is transferred to the perfusion apparatus. Here it is placed upon a small glass plate resting in a drainage funnel, a wire is hooked around the Y-shaped cannula and the pancreas raised so that the lower surface just rests upon the plate. The weight of the organ is supported by the cannula, and in turn by the piece of wire. The cannula is connected to the perfusion apparatus and no further attention is required until the perfusion period (usually one hour) has elapsed,

when the stopcock leading to the pancreas is shut off. During the first five or ten minutes of perfusion the pancreas increases enormously in size owing to the accumulation of fluid under the capsule and around the various lobules of the organ. From 300 to 500 cubic centimeters of fluid will be absorbed by a single pancreas, depending upon its size. At first the fluid drips off slowly from the surface of the pancreas and then runs in small streams from the openings of the veins.

3. Determination of weight of perfused pancreas. In order to make studies of the quantitative amounts of anti-diabetic substance obtained in different experiments it is necessary for us to know the weight of the portion of pancreas perfused. Although recently we have succeeded in perfusing entire organs we are always confronted with the fact that organs vary in weight and also with the possibility that we may not succeed in perfusing an entire organ. Occasionally a ligature catches a vessel which supplies a small lobe of the organ and sometimes a vessel becomes blocked mechanically by pressure of the overlying pancreas. By the method which we now use for suspending the pancreas we have practically succeeded in overcoming the latter difficulty.

The determination of the weight of the perfused pancreas offers another serious difficulty—we are dealing with organs containing varying amounts of fluid when the perfusion is completed. We cannot weigh the organs before perfusion is begun because we are not certain that every portion will be completely perfused.

The method which we have used previously in making comparative studies of the various factors involved in perfusion (7), (8) is the one used in this study. It is as follows: After perfusion is completed the pancreas is injected through the cannula with some easily distinguished coloring matter such as India Ink or Aniline Green (Naphthol-B). The colored portions of pancreas are cut away and weighed (after moderate squeezing in the hand to remove the great excess of fluid). These portions are then allowed to drain at room temperature over night, on a wire screen. They are then reweighed and the mean of the two different weights is used for the final weight.

In table 1 are shown the data from which the weight of pancreas was determined in perfusates 40, 41, 44 and 45 which are the ones used for special study in this paper. Twenty-four pancreases used in making these four perfusates had a total mean weight of 1186 grams giving an average weight of 49 grams per pancreas.

In order to test the accuracy of the method used in determining the weight of pancreas perfused we determined the weight of a series of unused pancreases for comparison. Twenty-three fresh pancreases were trimmed of fat, connective tissue and vessels (as was done with perfused pancreas before weighing) and the weights were found to be as follows: 43, 33, 53,

45, 52, 35, 42, 40, 43, 56, 25, 43, 69, 33, 57, 45, 48, 56, 57, 75, 47, 56 and 45 grams. These 23 pancreases had a total weight of 1098 grams giving an average weight of 47 grams per pancreas.

From these comparisons it seems evident that complete perfusion was accomplished and that no gross error could have been introduced by the method used in determining the weight of perfused pancreas since the crrespondence with the average fresh weight is remarkably close.

IV. PERFUSATES. The perfusates reported in this paper and used in the study of comparative yields by various extraction methods were all made with the apparatus shown in figure 1 and by the methods described above. The four selected are the ones which have been the objects of special study and represent the best yields which we have obtained by the perfusion method.

TABLE 1

Weight of perfused pancreas of pig

PERFUSATE NUMBER	NUMBER OF PANCREASES	WET WEIGHT OF PERFUSED PANCREAS	WEIGHT OF PERFUSED PANCREAS AFTER DRYING 24 HOURS	AVERAGE WEIGHT PER PANCREAS
		grams	*grams*	*grams*
40	4	157	120	35
41	8	447	372	51
44	6	317	259	48
45	6	398	303	58
Total.....	24	1319	1054	Av. 49

Protocols

Perfusate 40. Prepared May 15, 1923. Four pig pancreases, total mean weight of perfused portions 138 grams. Fluid used = N/20 HCl. Volume recovered 3660 cc. Temperature 39° to 43°C. Perfusion time 1½ hours. Volume passed through pancreases during perfusion = 81.7 liters.

Divided into two portions: 40-A (adjusted following day to pH of 4.1) and 40-B (first made faintly alkaline to litmus and then immediately adjusted to pH of 5.85). Very fine white precipitate formed in 40-B; filtered; clear pale yellow filtrate adjusted to pH 4.1.

Perfusate 41. Prepared May 21, 1923. Eight pig pancreases. Total mean weight of perfused portions, 410 grams. Fluid used = N/20 HCl. Volume recovered 3550 cc. Temperature 38° to 43°C. Perfusion time, 1½ hours. Volume passed through pancreases during perfusion = 229.4 liters.

Following day divided into four portions:

41-A adjusted to pH 4.1—no precipitate.

41-B adjusted to pH 5.85—copious finely divided white precipitate. Filtered rapidly leaving clear pale yellow filtrate. Adjusted to pH of 4.1 May 23, 1923.

41-C adjusted to neutrality (phenolphthalein). Heavy flocculent precipitate. Filtered rapidly. Filtrate pale yellow; readjusted to pH of 4.1.

41-D adjusted to neutrality (phenolphthalein) first then made distinctly alkaline to litmus. Precipitate which formed at first (like that of 41-C) went into solu-

tion. After standing 30 minutes HCl added carefully and precipitates formed at several points. These were removed separately and extracted with HCl (pH of 2.0).

Perfusate 44. Prepared August 14, 1923. Six pig pancreases, total mean weight of perfused portions 288 grams. Fluid used = N/20 HCl. Volume recovered 4500 cc. Temperature 43° to 45°C. Perfusion time, 1½ hours. Volume passed through pancreases during perfusion = 138.5 liters.

Following day adjusted to pH of 5.85, precipitate filtered off, and product adjusted to pH of 4.1, filtrate clear and straw colored. Precipitate extracted with HCl (pH 2.0).

Perfusate 45. Prepared August 16, 1923. Six pig pancreases, total mean weight of perfused portions = 350 grams. Fluid used = N/20 HCl Volume recovered 4380 cc. Temperature 41°C. Perfusion time, 1½ hours. Fluid supplied to pancreases during perfusion = 129 liters.

Following day adjusted to pH of 5.85, filtered off precipitate, and readjusted to pH of 4.1. Precipitate reëxtracted with HCl (pH of 2.0). August 18, 1923, perfusate readjusted to pH of 4.1.

The protocols state in detail the manner of handling the perfusates before they were injected into rabbits for assay. It is important to note that maximum precipitation is obtained in the process of neutralization at a pH of approximately 5.85 (electrometric). This differs very strikingly from the behavior of percolates and extracts. Before injection, however, perfusates are brought to the same reaction (pH 4.1) as for percolates and extracts (see below).

Table 2 shows the yield in rabbit units for each of the perfusates. Each of these has been tested several times of which the two highest are shown. In the last two preparations it was necessary to use a ratio of 1.25 grams of pancreas per two-kilo rabbit instead of the ratio of 2.5 grams of pancreas per two-kilo rabbit which is the one usually employed. Both of these were combinations of the original perfusate with the reëxtraction from the first precipitate and in testing the portions individually it was found that each produced nearly a rabbit unit when the 2.5 gram dose was used. Thus, in combining them, it became necessary to cut down the dose in order to keep the blood sugar drop within the allowable range.

In these preparations we can say that keeping for 27 days at pH of 4.1, on ice, caused no loss in potency.

As is shown in the table, dosage was regulated according to body weight and the absolute drop was used in figuring the rabbit units per kilo of pancreas used. The method of calculation has been explained in an earlier paper (9). The simplest formula is $14.28 \frac{a}{R}$ where a represents the number of milligrams fall in blood sugar in two hours and R the weight of pancreas representing a dose for a 2-kilo rabbit. The figures representing the units per kilo are accurate only to within about 10 per cent. The reason for this lies in the fact that the experimental error for the Folin-Wu (10) colorimetric method is about 7 mgm. for the samples used. With the

usual sample of 2.5 grams pancreas representing a dose, a reduction of 70 mgm. in the blood sugar would give 400 R.U. But a reading of 63 mgm. would give only 360 R.U.

As was explained above, it is sometimes necessary to change the dose factor, at times giving doses equivalent to 5 grams of pancreas, and at other times giving doses equivalent to 2.5 grams or even 1.25 grams. In

TABLE 2

Perfusates of pig pancreas

(Gravity perfusion under 200 mm. Hg pressure)

PREPARATION NUMBER	DATE 1923	AGE OF PREPARATION	PANCREAS: WEIGHT RATIO (R)	ANIMAL NUMBER	WEIGHT	FIRST BLOOD SUGAR	SECOND HOUR BLOOD SUGAR	DIFFERENCE (a)	R.U. PER KILO $\left(14.28\frac{a}{R}\right)$	AVERAGE YIELD R.U.	REMARKS
		days			*kilos*						
40−B*	5/17	2	2.5:2K	F-3	2.40	0.108	0.017	−0.091	521	492	Convulsions
40−B	6/11	27	2.5:2K	K-2	2.28	0.122	0.041	−0.081	463		1 hr. 50 min.
41B+P†	6/1	11	2.5:2K	H-6	2.20	0.090	0.010	−0.080	457	477	Convulsions 2 hrs.
41B+P	6/11	21	2.5:2K	K-3	2.16	0.113	0.026	−0.087	497		Convulsions 3 hrs. 27 min.
44B+P	8/21	7	1.25:2K	Z-2	2.84	0.112	0.048	−0.064	732	760	Precipitate
44B+P	8/21	7	1.25:2K	Z-3	3.07	0.092	0.023	−0.069	788		reëxtracted
45B+P	8/22	6	1.25:2K	Y-3	2.39	0.096	0.046	−0.050	572	675	Precipitate
45B+P	8/22	6	1.25:2K	Z-1	2.50	0.118	0.050	−0.068	778		reëxtracted
Average									601		

* "B" signifies a preparation adjusted to pH of 5.85.

† "P" signifies a preparation made by reëxtracting the precipitate from "B" with HCl (pH 2.0).

order to compare the results obtained it is necessary to calculate the total yield of preparations in terms of a common standard and for this purpose we have used the yield in rabbit units per kilo of pancreas.

The average yield of the eight tests shown in table 2 is 601 R.U.[2] per kilo.

[2] It should be borne in mind that this unit is at least three times the clinical unit adopted by the Insulin Committee of the University of Toronto, for it is the amount of insulin necessary to lower the blood sugar of a rabbit weighing 2 kgm., after 18 hours' starvation, 70 mgm. *in two hours*. The Toronto unit is one-third the amount necessary to produce a fall to 0.045 gram per 100 cc. *in five hours*.

V. PERCOLATION METHOD. Recently we have developed a method of obtaining insulin by reversed percolation, or upward filtration, of the pancreas. This method appears to possess many of the desirable features of perfusion while eliminating the bothersome details of ligation of cut vessels and the difficulty of weighing the pancreas, and also to possess certain advantages over the usual extraction method which we have employed.

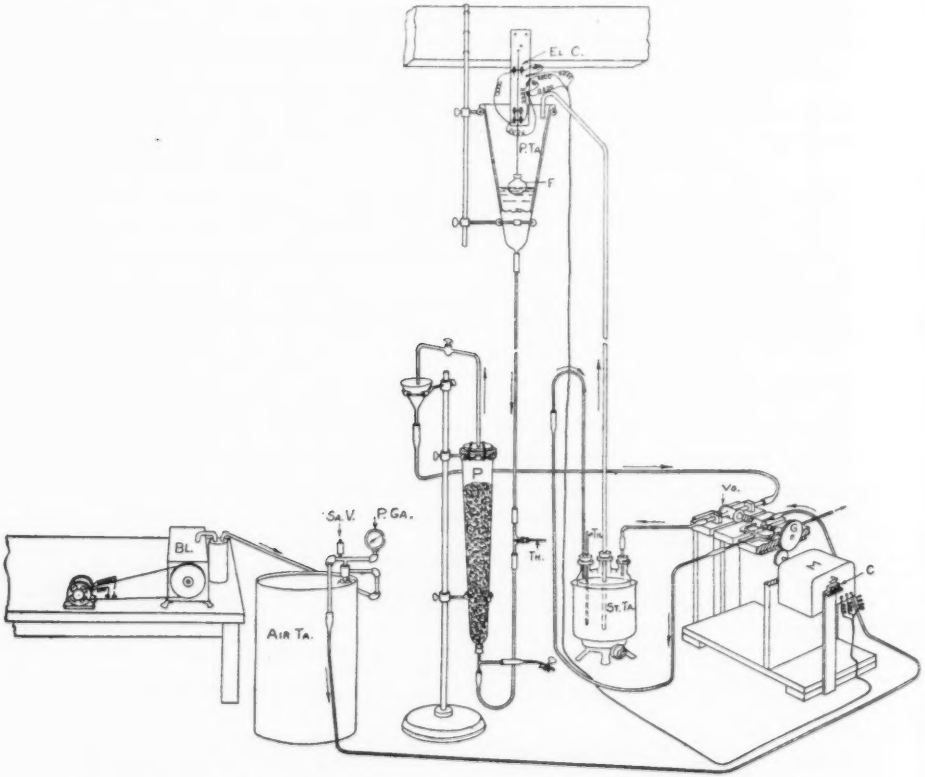

Fig. 2. Apparatus used for removal of insulin from macerated pancreas by reverse percolation. The percolator jars can be multiplied in number as many times as desired. Description in text.

1. *Apparatus.* The apparatus used for reversed percolation is shown in figure 2. In this method we have utilized the same essential principles for supplying heated fluid under constant gravity pressure as were used in the perfusion apparatus. The fluid is admitted to the bottom of the percolator, drains off from the top of the percolator into a funnel from which it flows by gravity into a storage and heating tank at a lower level, and is raised again by compressed air from the storage tank below to a pressure tank at a higher level.

a. Pressure tank. The pressure tank, *P. Ta.*, is of glass and at a height of 250 cm. above the top of the percolator, *P.*

b. Percolator. The percolator shown, *P.*, is 38 cm. high and is closed at the top by a large rubber stopper which is held in by heavy wires or better by a clamp. From the center of the stopper issues a glass tube containing a stopcock. Fluid enters at the bottom.

c. Drainage arrangements. Fluid draining from the top of the percolator flows into a glass funnel. From the funnel the fluid drains by gravity through the glass valve mounted on the valve shaft, *Vs.*, into the storage tank, *St. Ta.*

d. Storage tank. The storage tank, *St. Ta.*, has been described above. The same arrangement is used as was used for perfusion. The fluid is transferred from the lower tank to the upper tank by the same compressed air arrangement as was used in the previous apparatus.

2. Preparation of pancreas for percolation. Fresh pancreas is trimmed free of gross fat and connective tissue and macerated in a meat grinder. A weighed portion (250 grams for the size of percolator described above) is mixed with glass beads and placed in the percolator. The stopper at the top of the percolator is clamped securely and the stopcock at the top is closed. The stopcock is slowly opened until it is about half open and the fluid is allowed to flow through. Owing to the pressure inside the percolator the entire mass of pancreas and beads rises slightly and floats.

The fluid used is N/5 HCl kept at a temperature of 50°C. This is allowed to recirculate through the percolator for about two hours. The fluid obtained contains no gross particles of pancreas and appears to resemble closely the fluid obtained by perfusion except that in the case of percolation of pig pancreas it is slightly milky in appearance owing to the presence of fat, instead of the light straw color seen in pig pancreas perfusates. This milky appearance was not noted in the case of percolation of ox pancreas because it contains less fat. Proof that the milkiness is due to fat is found in the fact that a percolation of pig pancreas done with 80 per cent ethyl alcohol containing N/5 HCl did not show the milky appearance.

During the process of percolation there is very thorough aeration and continuous agitation of the fluid, good penetration of the pancreatic tissue, and a filtration of suspended material by the column of pancreas in the jar.

VI. PERCOLATES. Seven different percolates of pig pancreas are shown in table 3. The average yield, in rabbit units per kilo, of the tests made upon these products is 383. The highest yield shown by a percolate is 423 R.U. (33-C-A), the lowest is 360 R.U. or a difference of 15 per cent. Since we have an experimental error of 10 per cent, all of these yields must be regarded as very nearly identical.

Protocols

Percolate 6-A. Prepared June 6, 1923. Two hundred and fifty grams of macerated pig pancreas extracted in percolator 2 hours, 15 minutes with N/5 HCl at 47°C. Volume obtained 3410 cc. Volume of fluid supplied to pancreas = 56 liters. Immediately adjusted to pH 5.85 and small amount of flocculent precipitate formed which was filtered off. Filtrate clear. Adjusted the following day to pH of 4.1 and another precipitate formed. This was filtered off, extracted with acid water (pH 2.0) and tested 14 days later. Test not satisfactory owing to low initial blood-sugar level of rabbit (0.029 gram) and later tests not made owing to obvious spoiling of preparation. The last filtrate was tested on June 13 and June 16 with the checked results shown in table 3.

Percolate 30-C-B. Prepared July 16, 1923. Eleven hundred grams of macerated pig pancreas extracted in percolators 1½ hours at 46°C. with N/5 HCl; 4840 cc. obtained. Volume of fluid supplied to pancreas = 74 liters. This was adjusted to pH 4.1 immediately (with no precipitate formation) and allowed to stand over night. Following day a portion made neutral to litmus. White flocculent precipitate soon settled out. Filtered, giving clear, straw-colored filtrate which was readjusted to pH of 4.1.

Percolate 32-C-B. Prepared July 19, 1923. Twelve hundred and seventy grams of macerated pig pancreas extracted in percolators 1½ hours at 47° to 49°C. with N/5 HCl. 4650 cc. obtained. Volume of fluid supplied to pancreas = 73.6 liters. Solution stood in N/5 HCl for 18 hours when it was adjusted to pH of 4.1. After standing 18 hours at this acidity a portion was made neutral to litmus. Heavy precipitate formed which was removed by filtration and the filtrate was readjusted to pH of 4.1.

Percolate 33-C-A. Prepared July 20, 1923. Fifteen hundred and twenty-six grams of macerated pig pancreas. Extracted in percolators 1½ hours with N/5 HCl. Temperature 49°C. Volume of fluid obtained = 5630 cc. Volume of fluid supplied to pancreas = 173 liters. Preparation turbid (first noticed when it became necessary to remove part of the pancreas from one of the jars which was overfilled; solution contained small white flecks after this).

Divided into two portions, one of which (33-C-A) was adjusted to pH of 4.1. Heavy white flocculent precipitate formed which was not removed.

Percolate 36-C-B. Prepared August 1, 1923. Seven hundred and forty grams of macerated pig pancreas extracted in percolators 2¼ hours with N/5 HCl. Temperature 46°C. Volume of fluid obtained = 5480 cc. Volume of fluid supplied to pancreas = 112.5 liters. Solution slightly milky in appearance. Stood in N/5 HCl over night.

Following day made neutral to litmus when a small precipitate formed. Removed by filtration and reëxtracted with HCl (pH of 2.0). Filtrate readjusted to pH of 4.1.

Percolate 38-C. Prepared August 3, 1923. Eleven hundred and seventy-seven grams of macerated pig pancreas extracted in percolators 2 hours with N/5 HCl. Temperature 46° to 47°C. Volume of fluid obtained 4920 cc. Volume of fluid supplied to pancreas = 75.3 liters. Fluid quite turbid. Stood in N/5 HCl over night.

Following day adjusted to pH of 4.1 when a very heavy precipitate formed.

Eight days later a portion of this preparation was adjusted to neutrality (litmus). Grayish precipitate formed which was filtered off. Enough N/1 HCl was added to the first liter of filtrate to adjust the entire filtrate to pH of 4.1. When about one-fourth of the entire volume had filtered through, a 200 cc. portion of the filtrate was removed. This was centrifuged, the liquid decanted, and the small fine white precipitate which had formed spontaneously was taken up in 10 cc. of distilled water (proved toxic; see next paper).

Percolate 41-C-B. Prepared August 13, 1923. Nine hundred and thirty-four grams of macerated pig pancreas extracted in percolators 2½ hours at 43°C. with N/5 HCl. Volume of fluid obtained = 4430 cc. Volume of fluid supplied to pancreas = 92.6 liters. Preparation less turbid than previous percolates. Let stand in N/5 HCl over night.

Following day made neutral to litmus. Slight precipitate formed and was filtered off. Filtrate adjusted to pH of 4.1

TABLE 3

Percolates of pig pancreas

(Upward filtration under 250 mm. Hg pressure)

PREPARATION NUMBER	DATE 1923	AGE OF PREPARATION	PANCREAS: WEIGHT RATIO (R)	ANIMAL NUMBER	WEIGHT	FIRST BLOOD SUGAR	SECOND HOUR BLOOD SUGAR	DIFFERENCE (a)	B.U. PER KILO $\left(14.28\ \frac{a}{R}\right)$	AVERAGE YIELD B.U.
		days			*kilos*					
6-A	6/13	8	2.5:2K	J-2	2.28	0.115	0.049	−0.066	377	366
6-A	6/16	11	2.5:2K	M-4	2.28	0.101	0.039	−0.062	354	
30-C-B	7/18	2	2.5:2K	Q-3	2.50	0.130	0.070	−0.060	343	366
30-C-B	7/26	10	2.5:2K	S-1	1.93	0.127	0.059	−0.068	389	
32-C-B	7/26	7	2.5:2K	S-4	2.39	0.119	0.053	−0.066	377	360
32-C-B	7/31	12	2.5:2K	U-1	1.93	0.115	0.055	−0.060	343	
33-C-A	7/30	10	2.5:2K	T-5	2.96	0.133	0.063	−0.070	400	423
33-C-A	7/31	11	2.5:2K	Q-5	2.37	0.133	0.055	−0.078	446	
36-C-B	8/4	3	2.5:2K	T-2	2.72	0.171	0.105	−0.066	377	386
36-C-B	8/7	6	2.5:2K	V-4	1.82	0.137	0.068	−0.069	394	
38-C-I_2	8/13	10	2.5:2K	Y-2	2.61	0.110	0.049	−0.061	349	360
38-C-I_2	8/15	12	2.5:2K	X-5	2.28	0.116	0.051	−0.065	371	
41-C-B	8/15	2	2.5:2K	Y-2	2.61	0.126	0.045	−0.081	463	420
41-C-B	8/16	3	2.5:2K	Y-4	2.16	0.137	0.071	−0.066	377	
Average									383	

Attention may be drawn to the fact that no precipitate is formed in the process of neutralization of the extraction fluid, provided it is perfectly clear, until a reaction of approximately pH 7.0 is reached. When it is turbid a precipitate forms at a pH of 4.1 (protocols 33-C-A and 38C). The turbidity is caused by discrete particles of pancreatic material such as are found in large amount in the simple extracts and the precipitate which forms at pH 4.1 is largely composed of this material. Even when this precipitate has been removed, however, a further precipitation occurs, as described, at or near the neutral point. After removal of this neutral precipitate the fluid was adjusted to pH 4.1 (electrometric) before injection. Table 3 summarizes results with seven different percolations of pig pancreas.

VII. Simple extraction. The early demonstration in this laboratory (11), (12), (13) that boiling in acid medium does not destroy insulin and that heating to 80°C. (14) often increases the potency of a given preparation (probably by removal through coagulation of interfering substances) led us to adopt about April first of this year the practice of making all our insulin with the aid of heat. As noted in the previous sections water acidulated with HCl and heated to approximately 50°C. is used in both the perfusion and the percolation methods. Higher temperatures offer no advantages with these methods. In simple extraction, however, better yields have been obtained by heating to 75 to 80°C. for a short time than heating to 50°C. for one to two hours.

Simple extraction is accomplished by adding the freshly macerated material from carefully trimmed pancreas (pig only has been used in this study) to four volumes of 0.2 N HCl and heating with continuous stirring in an enamelled vessel over a free flame, or heating (with occasionally shaking) in a glass flask immersed in a water bath. Close attention has been given to the temperature and time of heating so as to find the conditions which give a maximum yield of insulin in the crude extract. Once the material has been thoroughly heated, say to 75°C., there seems to be no material advantage in prolonging the heat. With the water in a bath already at 80°C. and a low flame a suspension of 500 grams macerated pancreas with 2 liters of extracting fluid can be brought to 75°C. within ten minutes. Starting with the water cold the time for heating in a flask to this point over a high flame is not more than fifteen to twenty minutes. The chief advantage of the flask is that the contained material can be cooled more readily and this facilitates removal of the fat by straining of the pulp through cheese cloth and muslin.

After titration of one or two samples the strained material is next neutralized to a pH of 4.1 (electrometric) which has been found to result in most rapid filtration and, fortunately, also to give a yield of insulin in the filtrate which is maximal on the second day (6).

Reëxtraction. On numerous occasions, during the first year of work upon aqueous extraction of insulin, it was noted that a second extraction with cold acidulated water gave a stronger preparation than the first and in some cases good products were obtained from a third extraction. For a time alcohol was used in the second extraction until we became convinced that water gave better results just as we had previously been convinced of the superiority of water for first extraction. Reëxtraction of the first precipitate (of acid metaproteins) with 80 per cent alcohol rarely gave any additional yield of any consequence; but reëxtraction of the precipitate with water acidulated with HCl to pH 2.0 has, on several occasions *more than doubled the yield* in the filtrate. Until this observation was made (about June 15, 1923) the yield of crude insulin by both perfusion and

percolation had been distinctly greater than by simple extraction.[3] Re-extraction of the corresponding precipitates after perfusion had also yielded increased potency about the same time; while after percolation there has never been any increase from reëxtraction of the first precipitate. The reasons for these differences in behavior will be discussed in the next paper, on the release of insulin from the proteins of the pancreas.

Protocols

Extract 1. Prepared May 14, 1923. One hundred and fifty grams of macerated pig pancreas previously trimmed of fat and connective tissue placed in 4 volumes of N/5 HCl and allowed to stand over night at room temperature. Following morning brought up to 75°C. in water bath (10 minutes) and immediately cooled in running water.

Adjusted to pH of 4.1 and filtered. Filtrate clear and pale yellow.

Extract 2. Prepared May 14, 1923. One hundred and fifty grams of macerated pig pancreas (trimmed) placed in 4 volumes of N/5 HCl. Brought up to 75°C. in water bath (required 7 minutes) and cooled immediately in running water. Reaction then adjusted to pH of 4.1 and the precipitate which formed was filtered off. Filtrate clear and of pale lemon color.

Extract 3. Prepared May 25, 1923. Five hundred grams of hashed pig pancreas (previously trimmed of fat and connective tissue) extracted four times with 1250 cc. of N/10 HCl by heating to 75°C. and filtering through double layers of gauze. Total volume of extracting medium = 5000 cc. Entire volume adjusted to pH of 5.85 giving very heavy precipitate. Filtered very rapidly, leaving clear lemon-yellow filtrate.

Extract 7-D. Prepared June 5, 1923. Two hundred and fifty grams of macerated pig pancreas placed in four volumes of N/5 HCl and kept on ice over night. The following day it was heated (with continuous stirring) in an open jar at an average temperature of 52°C. for 2½ hours.

Enough N/5 HCl added to one portion (7-D) to give dilution of 1:10. Filtered very slowly. Filtrate made neutral to litmus. Heavy precipitate formed; solution filtered very rapidly and clear. Filtrate adjusted to pH of 4.1.

Extract 40-E. Prepared August 8, 1923. Eight hundred grams of trimmed and macerated pig pancreas placed in 4000 cc. N/5 HCl. Heated to 50°C. and kept at this temperature for one hour with frequent stirring. Filtered through double layer of gauze. Filtrate adjusted to pH of 4.1.

Extract 41-E. Prepared October 18, 1923. Eight hundred grams of trimmed and macerated pancreas in 3200 cc. 0.2 N HCl heated to 50°C. for one hour, with frequent stirring. Strained. Neutralized to pH 4.1 (titration to phenolphthalein). Precipitate reëxtracted with 1000 cc. acidulated water (pH 2.0).

Extract 43-E. Prepared November 3, 1923. Six hundred and seventy-five grams of trimmed and macerated pig pancreas in 2700 cc. 0.2 N HCl heated in water bath to 75°C. Required 15 minutes, cooled to room temperature, strained, neutralized to pH 4.16 (electrometric). Precipitate reëxtracted with 2000 cc. acidulated water (pH 2.0) but tested at pH 4.1.

Extract 44-E. Prepared November 12, 1923. Five hundred and fifty grams of trimmed and macerated pig pancreas in 2200 cc. 0.2 N HCl heated in water bath to

[3] The figures given in previous communications (7), (8) upon a comparison of perfusion, percolation and extraction apply only to the first filtrate without taking into account the extra yields from reëxtraction of the first precipitate.

TABLE 4

Simple extracts of pig pancreas

PREPARATION NUMBER	METHOD	DATE OF TEST 1923	AGE OF PREPARATION	PANCREAS: WEIGHT RATIO (R)	ANIMAL NUMBER	WEIGHT	FIRST BLOOD SUGAR	SECOND HOUR BLOOD SUGAR	DIFFERENCE (a)	R.U. PER KILO $\left(14.28\frac{a}{R}\right)$	AVERAGE YIELD R.U.	REMARKS
			days			*kilos*						
1	N/5 HCl (4 vols.) room temp. 24 hrs. then heated to 75°C. for 10 min.	5/21	7	5:2K	E-1	3.50	0.070	0.015	−0.055	157		Convul. 2½ hrs. Died 20 min. later
2	N/5 HCl (4 vols.) heated to 75°C. and stirred; then allowed to cool	5/16	2	5:2K	E-5	2.40	0.143	0.080	−0.063	180	164	
		5/18	4	5:2K	E-2	2.80	0.095	0.043	−0.052	148		
3	N/10 HCl (10 vols.) Four separate extractions with 2½ vols. each heated to 75°C.	5/26	2	5:2K	F-2	2.72	0.090	0.030	−0.060	171	183	
		5/29	5	5:2K	H-2	2.00	0.089	0.021	−0.068	194		Convulsions 2½ hours
7-D	N/5HCl (4 vols.) on ice for 20 hrs. then heated and stirred at 50°C. 2½ hrs. Ppt. reëxtracted with 500 cc. HCl pH 2.0	6/12	6	2.5:2K	H-2	2.04	0.088	0.033	−0.055	314	274	Convulsions 2 hrs. 18 min.
		6/14	8	2.5:2K	M-2	2.50	0.074	0.033	−0.041	234		Convulsions 2 hrs.
		6/20	14	2.5:2K	J-2	2.28	0.106	0.056	−0.050	286		
40-E	N/5 HCl (5 vols. heated to 50°C. for 1 hour with stirring*	8/9	1	2.5:2K	Y-1	3.07	0.105	0.045	−0.060	343	306	
		8/10	2	2.5:2K	U-4	2.16	0.120	0.073	−0.047	269		
											Total	
41-E	N/5 HCl (4 vols.) heated to 50°C. 1 hr. Neutr. to pH 4.1 Precip. reëxtr. with 1000 cc. HCl pH 2.0	10/20	2	2.5:2K	Z-34	1.70	0.109	0.054	−0.055	294	614	
					Z-32	1.47	0.082	0.035	−0.047			
					A-6	1.36	0.107	0.054	−0.053			
		10/25	7	2.5:2K	B-2	2.49	0.109	0.054	−0.055	320		
					A-5	1.57	0.114	0.057	−0.057			

43-E	N/5 HCl (4 vols.) heated to 75°C. Neutr. to pH 4.16	11/6	3	2.5:2K	A-6	1.36	0.098	0.043	−0.055	360	
					D-1	2.09	0.175	0.103	−0.072		
		11/12	9		B-2	2.15	0.143	0.080	−0.063		1080
	Ppt. rëxtracted with 2000 cc. HCl pH 2.13	11/7	4	1.25:2K	A-4	1.58	0.129	0.066	−0.063	720	
					C-5	1.47	0.105	0.036	−0.069		
					D-4	3.06	0.112	0.056	−0.056		
44-E	N/5 HCl (4 vols.) heated to 75°C. Neutr. to pH 4.18	11/15	3	2.5:2K	D-2	2.72	0.120	0.035	−0.095	480	
					E-2	2.60	0.129	0.035	−0.074		1180
	Ppt. reëxtracted with 2200 cc. HCl pH 1.97	11/15	3	1.25:2K	C-5	1.47	0.093	0.033	−0.060	700	
		11/16	4		C-3	2.00	0.115	0.062	−0.053		

* The reëxtraction of this precipitate was lost.

75°C. Required 20 minutes. Cooled to room temperature, strained and neutralized to pH 4.18 (electrometric). Precipitate reëxtracted with 2000 cc. acidulated water pH 2.00 and tested at pH 4.1.

The several preparations given in table 4 show some of the variations of the method which have been used in preparing extracts. In all of the cases shown, the first step was maceration of the pancreas in a meat grinder. The main procedures are shown in the protocols. Preparation 2 proved to be as potent as preparation 1. Extraction with heat effected a saving of many hours.

In making preparation 3 the effect of four separate extractions with a total volume of solution comparable to that generally used in perfusion was studied. The yield by this method proved to be practically the same as that obtained by the two previous methods.

The next preparation (7-D) was made by modification of some of the factors utilized in percolates. The pancreas was kept at 50°C. for 2½ hours with continuous stirring. The yield in this case was definitely greater than by previous methods.

TABLE 5

Largest yields of crude insulin from pig pancreas

METHOD	LARGEST YIELD R.U. PER KGM. OF PANCREAS	AVERAGE YIELD PER KGM.		REMARKS
		R.U.	Clin. U.	
Percolation	423	383	1149	Ppt. reëxtracted
Perfusion	521	484	1452	
	788	717	2151	Ppt. reëxtracted
Simple extraction	343	232	696	
	1800	1000	3000	Ppt. reëxtracted

Preparations 40-E and 41-E were made by a simplification of the methods used in preparing 7-D, by shortening the time of heating to one hour.

Preparations 43-E and 44-E were made by heating the suspension of pancreas in 0.2 N HCl only just to 75°C. and immediately cooling. Neutralization after straining was carried out with great care and in both cases resulted in hitting the exact end-point desired. The yield from the first filtrate is greater than that obtained in the case of 40-E and 41-E. The difference must be due to the different manner of heating, for all the other conditions were identical.

The preceding table summarizes the best results by each of the three methods of extraction.

For comparison with the yields recently reported by Best and Scott (15) it is necessary to multiply the figures in column 3 of the table by three. This has been confirmed by injection of iletin (Insulin, Lilly) into rabbits and estimating the strength by the Rochester unit.

SUMMARY AND CONCLUSIONS

1. Three distinct methods of treating the pancreas of slaughter-house animals with aqueous solutions for extraction of insulin are described, namely, perfusion, percolation and simple extraction.

2. For comparison in all three methods pig pancreas was treated with water acidulated with hydrochloric acid and heated to 50°C. In perfusion the strength of acid found most satisfactory is 0.05 N; for percolation and simple extraction, 0.2 N. Finally, for simple extraction heating just to 75°C. and immediately cooling has proved more satisfactory than heating to 50° for one hour.

3. In all cases the fluid obtained after merely straining is neutralized to a certain reaction varying with the method of extraction. This throws down a heavy precipitate of acid metaproteins.

4. In agreement with earlier preliminary results on the comparison of the three methods, the yield of crude insulin *in the first filtrate* is greatest after perfusion, next greatest after percolation and least after simple extraction. This depends upon a difference in the kind and amount of protein extracted from the pancreas cells.

5. By reëxtraction of the first precipitate after perfusion and simple extraction, but not after percolation as here carried out, it is possible to increase the yield of crude insulin enormously.

6. The yield of crude insulin is expressed in Rochester units, i.e., the amount of insulin necessary to lower the blood sugar of a rabbit, which has been fasted 18 hours, 70 mgm. in 2 hours. This unit is at least three times as great as the unit adopted by the Insulin Committee of the University of Toronto.

7. The largest yield of crude insulin is well over 1500 Rochester units, or more than 4500 Toronto units, per kilogram of fresh pancreas.

BIBLIOGRAPHY

(1) Clark: Jour. Exper. Med., 1916, xxiv, 621.
(2) Landes, Garrison and Moorhead: Arch. Int. Med., 1922, xxix, 852.
(3) McCarthy and Olmstead: This Journal, 1923, lxv, 252.
(4) Murlin, Clough and Stokes: This Journal, 1923, lxiv, 348.
(5) Murlin, Clough, Gibbs and Stone: This Journal, 1923, lvi, 253.
(6) Piper, Allen and Murlin: Journ. Biol. Chem., 1923, lviii, 321.
(7) Clough and Murlin: Proc. Soc. Exper. Biol. and Med., 1923, xx, 417.
(8) Clough, Allen and Murlin: Proc. XIth International Congress of Physiologists, Edinburgh, Quart. Journ. Exper. Physiol., 1923, Suppl., Vol. 88.

(9) CLOUGH, ALLEN AND ROOT: This Journal, 1923, lxvi, 461.
(10) FOLIN AND WU: Journ. Biol. Chem., 1919, xxxviii, 81.
(11) MURLIN, KRAMER AND SWEET: Journ. Metab. Res., 1922, ii, 19.
(12) ALLEN, PIPER, KIMBALL AND MURLIN: Proc. Soc. Exper. Biol. and Med., 1923, xx, 519.
(13) MURLIN, CLOUGH, GIBBS AND STOKES: Journ. Biol. Chem., 1923, lvi, 253.
(14) PIPER, MATTILL AND MURLIN: Proc. Soc. Exper. Biol. and Med., 1923, xx, 413.
(15) BEST AND SCOTT: Journ. Biol. Chem., 1923, lvii, 709.

A STUDY OF THE MECHANISM OF CHANGE IN RESISTANCE OF ERYTHROCYTES TO HYPOTONIC SALT SOLUTION[1]

I. The Effect of Artificial Anemia, and of the Treatment of Red Cells with Various Blood Protein Fractions

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In the solution of the larger problem of the process of physiologic blood destruction, which is fundamental to any real understanding of the "blood diseases", a knowledge of the process by which changes in resistance of the red blood corpuscle to hypotonic salt solution are brought about would seem to have a part. A mass of data on the resistance of the red blood corpuscle, accumulated from clinical studies, is available (5), (6), (7). The point of outstanding interest in these data is that, with the exception of hemolytic jaundice, all pathologic conditions which produce any marked change in the resistance of the corpuscles to hypotonic salt solution cause an increase in resistance. Anemias, both primary and secondary, although they may produce a slight increase in fragility, with usually an increase in the range over which hemolysis takes place, tend to increase resistance. In pneumonia and in diabetes there are increases which are independent of anemia. In obstructive jaundice, and in poisoning from lead, ditolucle, phenyl-hydrazine and "oestrin," (10) there are marked increases of resistance. On the other hand, hemolytic jaundice is accompanied by a definite increase in fragility, without the simultaneous appearance of more resistant cells. The fact that because of its ease of performance we already have a great mass of clinical data correlated with this test, and also that in one clinical entity of which rapid blood destruction is the outstanding feature, there has been found a consistency in the reaction of the corpuscles to this test, calls for an understanding of the test. It seems reasonable to hope that when the factors which determine whether corpuscles are more or less resistant to hypotonic salt solution are understood, some light will be shed on the clinical conditions in which these changes in resistance have occurred, and also, possibly, on the physiologic process of blood destruction.

[1] In collaboration with the Clinical Section of Dr. H. Z. Giffin.

Although a certain amount of clinical data has accumulated in correlation with other methods of testing changes in the corpuscle, because of the extreme complexity of the problem, attention has been confined to the one test, lysis by hypotonic salt solution. The mechanism of lysis and hemolysis, on a knowledge of which an understanding of changes in resistance depends, has as yet not been satisfactorily determined, although work such as that of Bechold (2) is in the direction of elucidating the matter. The reversible hemolysis phenomenon, first demonstrated by Rohonyi (9) and repeated recently in a more convincing manner by Brinkmann (3) has forcibly demonstrated our ignorance with regard to the mechanism of hemolysis. The problem of determining the factors which cause a change in the resistance of the red cell to lysis and hemolysis must, since the mechanisms of lysis and hemolysis themselves are not understood, necessarily be a complicated one, and it is imperative to reduce it to its simplest denomination.

I have attacked this problem of the mechanism of change in the resistance of corpuscles to hypotonic salt solution by the obvious method of attempting to produce changes in resistance in vitro by agents that might conceivably be factors in vivo. The work has been confined to blood from human beings. In approaching the problem two outstanding points attracted my attention; that hypocytosis and increased resistance are often associated, and that increased resistance is likely to be found in conditions in which, because of an increase in the serum albumin fraction an increase in an antiferment element of the blood might be suspected, or in which because of an increase in blood fats a protection against ferments might be inferred. The problem was approached along two main lines: *a*, an attempt to determine whether in vitro increase in resistance results from hypocytosis, and *b*, the effect of prolonged exposure of corpuscles to the influence of the different blood protein fractions and to serum from which the supposedly enzyme inhibitory constituents had been removed was investigated.

EFFECT OF INCREASING THE PROPORTION OF PLASMA TO CORPUSCLES. To simulate the blood conditions of certain anemias, a hypocythemia was produced in vitro by diluting citrated blood with native plasma. These mixtures were incubated at 37° and in the ice box, and were tested for sterility at the end of the incubation period. After periods of incubation, varying from one to nine days, resistance tests were made with 0.9 per cent sodium chlorid. This, when diluted through a scale of twenty-six tubes, the first of which contains water, gives an interval of 0.036 per cent. For convenience, only the first two figures are given. The readings are on the basis of 0 to 4, 4 being complete hemolysis. Very slight hemolysis is recorded as a trace (table 1). It will be seen that there are two factors at work; one causes the corpuscles to increase in resistance, the

other causes them to deteriorate and to increase in fragility, as seen by the coincident movement of initial hemolysis to lower dilutions. The tendency to increase in resistance is greater in those corpuscles that are in a geater volume of plasma and at the higher temperature.

In a second experiment, blood and plasma were mixed in the following proportions: whole blood, one unit of blood to one-half unit of plasma, one

TABLE 1

Test of relative change in resistance of corpuscles to hypotonic salt solution on incubation in greater and less amounts of their own plasma at 37°C. and at icebox temperature

INCUBATED IN	DAYS INCUBATED	READING OF RESISTANCE TEST, PER CENT SODIUM CHLORID												
		0.08	0.11	0.15	0.18	0.22	0.25	0.29	0.33	0.36	0.40	0.43	0.47	0.51	0.56
Blood plus plasma in incubator	0						4	3	2	trace	0				
	1					4	3	3	2	2	2	1	1	trace	
	2			4	3	3	3	2	2	2	1	1	1	1	trace
	3			4	3	3	2	2	2	1	1	1	1	1	1
	6		4	3	3	3	3	2	2	2	2		1	1	1
Blood minus plasma in incubator	0						4	3	2	trace	0				
	1					4	3	3	3	2	2	1	1	1	0
	2					4	3	3	2	2	1	1	1	1	trace
	3							4	3	3	2	1	1	1	
	6							4	3	3		2			1
Blood plus plasma in icebox	0						4	3	2	trace	0				
	1					4	3	3	3	2	1	trace	0		
	2			4	3	3	3	2	2	2	1		0		
	3			4	3	3	3	2	2	2	1	1	trace		0
	6			4	3	3	3	2	2	2	1	1	1	1	trace
	9			4	3	2	2	2	2	1	1	1	1	1	1
Blood minus plasma in icebox	0						4	3	2	trace	0				
	1				4	3	3	2	2	2	1	1	trace	0	
	2			4	3	3	2	2	2	2	1	1	trace		0
	3		4	3	3	3	3	2	2	2	1	1	trace		0
	6			4	3	3	3	3	2	2	2	1	1	1	1
	9						4	3	3	3	2	2	2		1

unit of blood to two units of plasma, and one unit of blood to five units of plasma. These mixtures were placed in the incubator for three days. The resistance was found to be proportional to the amount of plasma present. It would appear that under these conditions resistance of the corpuscles to hypotonic salt solution increases with a simultaneous increase in the range over which hemolysis takes place, simulating in this respect

the state of the corpuscles found in certain clinical conditions in which there is anemia.

As there was always a lag in the increase of resistance of the corpuscles on incubation, tests were made to see whether this time element was due to either the development or destruction of some element of the plasma. Corpuscles were incubated in plasma which had been previously incubated with and without corpuscles, and in order to test especially the possibility of deterioration of complement being a factor, blood was incubated in fresh plasma, in inactivated plasma and in plasma to which guinea-pig serum had been added in the proportion of 1 to 12. In all these tests there was no difference in degree in the rate at which the corpuscles increased in resistance.

The possibility was considered that changes in resistance might be due to the development of a lack of oxygen, since the corpuscles which increased their resistance in larger amounts of plasma were, after settling to the bottom of the tube, further away from their oxygen supply than corpuscles in less plasma. This point was tested by incubating the same proportions of corpuscles and plasma in tall narrow columns, and in low broad ones: also by comparing the effect of repeated aeration with the effect of removal of oxygen by pyrogallic acid. There were no differences in the rate of increase in resistance that could be attributed to the oxygen supply

Thus far it appears that corpuscles increase their resistance under the condition of artificial anemia, that this increase takes time, that it can occur at temperatures below body temperature although less rapidly, that lack of oxygen does not produce it, and that the time factor is not due to the production or destruction of something which, when produced or destroyed, causes the increase in resistance. Time itself is the necessary element in changing the corpuscles. On incubating corpuscles in a deficiency of plasma, deterioration takes place more rapidly than the resistance increases.

THE EFFECT OF THE BLOOD SERUM FRACTIONS ON RESISTANCE. As I have shown (1) and as has been verified by Wearn, Warren and Ames (11), the red corpuscles of man are capable of withstanding the wear and tear of the circulation of man for a period of three months. Since from the mechanical standpoint this is to me rather incredible, the possibility seemed worth considering that, regarding the blood as a tissue, there might be in the plasma some specific enzyme mechanism comparable to that which has been suspected of being responsible within the nucleated cell for growth or autolysis of the cell substance, and that this mechanism could build up certain constituents of the red corpuscle and keep it in repair. If, then, the synthetic activity of the enzyme mechanism had been excessive, corpuscles of increased resistance to hypotonic salt solution because of their

stronger stroma would be the result. On the basis of Robertson's "reciprocal catalysis" it was conceived that if there were such a mechanism, it might be reversible, and that less resistant corpuscles might result from exposure to the enzyme mechanism in its destructive phase, and that changes in the resistance of the red cells to hypotonic salt solution might result from any shift from neutrality of this hypothetic enzyme anti-enzyme balance. It was assumed that the synthetic phase of such a mechanism would be associated with the albumin fraction of the serum and its lytic phase with the globulin fraction.

It was also conceived as a possibility that corpuscles in the course of their life in the circulation might at times be excessively exposed to the action of proteolytic or lypolytic enzymes absorbed from the intestinal tract, or elsewhere, because of insufficient protection from corresponding anti-enzymes, or from serum lipoids, and that this would result in the appearance of fragile corpuscles; or, if protection had been maintained more consistently than was normal, an average resistance greater than normal would result. Some excuse for this hypothesis is found in a certain amount of correlation between increased resistance and some clinical conditions in which there is likely to be found an increased serum lipoid content, or with which increase in the proportion of the serum albumin fraction is likely to be associated.

An attempt was made to investigate these possibilities by subjecting corpuscles to the effects of the euglobulin, globulin and albumin fractions of the plasma, and to the effects of plasma treated with kaolin, carbon dioxid, and deprived of lipoids.

As a preliminary experiment, the effect of commercial preparations of trypsin and steapsin on the fragility of red blood corpuscles was tested. Solutions of isotonic sodium chlorid were made, containing 1 per cent, 0.5 per cent, 0.25 per cent, and 0.0625 per cent trypsin, and the reaction was adjusted to pH 7.4. To 10 cc. of these solutions 1 cc. of blood was added, and the whole incubated for one and one-half hours, after which hemolysis was evident in the tubes containing 0.5 per cent and 1.0 per cent trypsin, but not in the others. After removing the media, the resistance of the corpuscles was tested with a scale of hypotonic salt solutions (table 2). It was found that within a range of concentration of the enzyme which did not produce hemolysis on incubation, an increase in fragility of the corpuscles had occurred. Similar results were obtained with steapsin.

The effect of the albumin fraction. To obtain the albumin fraction, the globulin fraction was precipitated either from fresh serum, or from citrated plasma by half saturation with ammonium sulphate. The precipitated globulin was separated by centrifuging from the albumin-containing solution, and each was dialyzed in an S and S 579A diffusion cup against running water until the ammonium sulphate had disappeared to such an

extent that the solutions gave about the same precipitate with barium chlorid that was given by tap water. This took from three to four days. The solutions were then made up to isotonicity with sodium chlorid and the hydrogen-ion concentration adjusted with the aid of the phenolsulphonephthalein commercial color scale. Mixtures of blood and the solutions were incubated. The action of fractions from serum of the same group as the corpuscles used, from serum of a different group, from serum of a

TABLE 2

Test of change of resistance of corpuscles to hypotonic salt solution after incubation for one and one-half hours at 37°C. in trypsin solutions

INCUBATED IN TRYPSIN	READING OF RESISTANCE TEST, PER CENT SODIUM CHLORID												
	0.29	0.33	0.36	0.40	0.43	0.47	0.51	0.54	0.61	0.68	0.76	0.83	0.90
per cent													
0.0	4	3	3	2	1	1	trace	0					
1.0										4	3	2	1
0.5									4	3	2	1	1
0.25						4	3	3	2	1	1	trace	trace
0.125					4	3	3	2	1	trace	0		
0.0625	4	3	3	3	3	1	1	trace	0				

TABLE 3

Test of changes of resistance of human corpuscles to hypotonic salt solution after incubation in isotonic sodium chloride solutions, the albumin fraction of human serum, and 10 per cent egg albumin

INCUBATED IN	HOURS INCUBATED	READING OF RESISTANCE TEST, PER CENT SODIUM CHLORID										
		0.15	0.18	0.22	0.25	0.29	0.33	0.36	0.40	0.43	0.47	0.51
Sodium chlorid	4				4	3	2	2	1	0		
	18				4	3	2	2	1	1	1	0
Albumin fraction	4				4	3	2	2	1	0		
	18	2	2	1	1	1	1	1	trace	trace	trace	trace
Egg albumin, 10 per cent	4				4	3	2	2	1	0		
	18							4	2	2	2	1

different species, and the effect of a non-treated foreign substance, egg albumin, were tested.

The following protocol (table 3) illustrates the result with the albumin fraction of serum of the same group as the corpuscles used, and with egg albumin diluted with isotonic chlorid. The albumin fraction of group II serum was dialyzed for four days and adjusted to approximately pH 7.6. Eight-tenths of a cubic centimeter of group II blood was added to 3 cc. of

each of the solutions used. In eighteen hours there was a slight deterioration of the corpuscles in sodium chlorid solution with no increase in resistance. In the albumin fraction there was very marked increase in resistance with slight deterioration, as seen by traces of hemolysis in the lower dilutions. Corpuscles in 10 per cent egg albumin became less resistant.

The supposition that the increase in resistance of the corpuscles, when incubated in the albumin fraction of serum from the same species, and of a like group, was due to some biologic activity, would seem to be ruled out by the fact that the same results were produced by incubation of human corpuscles in dog and beef albumin (table 4). With three days' incubation

TABLE 4

Test of changes in resistance of human corpuscles to hypotonic salt solution after incubation in beef serum albumin and dog serum albumin

HUMAN CORPUSCLES IN	DAYS INCUBATED	READING RESISTANCE TEST, PER CENT SODIUM CHLORID												
		0.11	0.15	0.18	0.22	0.25	0.29	0.33	0.36	0.40	0.43	0.47	0.51	0.54
Human serum	0					4	2	1	0					
Beef serum albumin	2	4	2	1	1	1	1	1	1	1	1	1	1	1
Dog serum albumin	2	4	1	1	1	1	1	1	1	1	1	1	1	1

TABLE 5

Test of the increase in resistance of human corpuscles to hypotonic salt solution on incubation in a 1:10 mixture of blood and isotonic sodium chloride solution

DAYS INCUBATED	READING RESISTANCE TEST, PER CENT SODIUM CHLORID																
	0.03	0.06	0.09	0.12	0.15	0.18	0.21	0.24	0.27	0.30	0.33	0.36	0.39	0.42	0.45	0.48	0.51
0										4	3	2	1	1	tr.	0	
1										4	3	2	1	1	1	tr.	0
2					4	3	3	3	3	3	2	2	2	1	1	1	1
3	4	2	1	1	1	1	tr.*	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
4					4	3	2	2	2	1	1	1	1	1	1	1	1

* tr. = trace.

instead of two, human corpuscles in a 1 to 10 mixture of blood and isotonic sodium chlorid solution increased their resistance similarly (table 5). Furthermore, washed corpuscles incubated in isotonic sodium chloride solution, in four days showed a similar degree of increase in resistance (table 6). As the increase in resistance eventually takes place in the total absence of serum, it would seem improbable that the increase in resistance which takes place in the presence of the albumin fraction, can be due to that fraction, although it would seem that the serum constituent has an accelerating effect on the progress of the increase in resistance of red corpuscles.

The question of a fragility-producing enzyme. To detect the presence of an enzyme that would so affect the corpuscle that an increase in fragility to hypotonic salt solution resulted, the globulin fraction of plasma (obtained as previously described) was incubated with blood. Instead of an increased fragility, an increase in resistance resulted, comparable to that obtained with the albumin fraction. The globulin fraction from the serum of an animal of another species, after dialyzation for four days similarly did not cause an increase in fragility.

As it is possible that in the long dialyzation necessary to remove the ammonium sulphate any enzyme originally present might have deteriorated, a euglobin fraction was made by diluting serum and saturating it with carbon-dioxid. The precipitate thus obtained was dissolved in isotonic sodium chloride solution, and its activity tested both at the hydrogen-ion concentration of the blood, and at that given by saturation with carbon-dioxid. Corpuscles incubated in the globulin fraction that

TABLE 6

Test of the increase in resistance of washed human corpuscles to hypotonic salt solution on incubation in isotonic sodium chloride solution

DAYS INCUBATED	READING OF RESISTANCE TEST, PER CENT SODIUM CHLORID																
	0.04	0.08	0.11	0.15	0.18	0.22	0.25	0.29	0.33	0.36	0.40	0.43	0.47	0.51	0.54	0.58	0.61
0									4	3	3	2	1	0			
1								4	3	3	3	2	2	1	1	1	0
2						4	3	2	2	2	2	1	1	1	1	1	1
3							4	3	3	2	2	2	1	1	1	1	1
4	4	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1

was acidified, and those that were incubated at alkaline reaction increased in resistance. Under the influence of carbon dioxid the change was accomplished in two hours. At the hydrogen-ion concentration of the blood, the rate of increase in the presence of the euglobin fraction was a little more rapid than in a 1 to 10 mixture of blood and saline.

To eliminate entirely any effect of the plasma, twice washed human corpuscles were incubated in the euglobin fraction obtained from 20 cc. of human serum diluted to 10 cc., 2 cc. of corpuscles being added. At the same time the effect of beef euglobulin was tested and also, as a control of the result obtained with acidified euglobulin, washed corpuscles were incubated in saline through which carbon dioxid had been bubbled until the corpuscles were a dark red. Upon testing with hypotonic salt solution after eighteen hours' incubation, the corpuscles that had been in the human euglobulin solution had increased moderately in resistance, those in beef euglobulin had decreased in resistance, and those that had been treated with carbon dioxid had increased profoundly in resistance (table 7).

The globulin fraction does not appear to contain an enzyme capable of causing autogenous increase in fragility. Since carbon dioxid causes an increase in resistance of washed corpuscles, its action must be direct and not due to any activating effect on a blood constituent. The increase in resistance on saturation with carbon dioxid is undoubtedly due to a different mechanism from that which comes into play when the corpuscle is subjected to low tensions of carbon dioxid, such as were employed by Doisy and Eaton, who found an increase in fragility due to an osmotic pressure change caused by a shift between the plasma and corpuscle chlorid and carbonate.

It was still possible that in normal serum there might be an enzyme present that would become active with increased hydrogen-ion concentration. Gas from a carbon dioxide tank was bubbled through whole bloods for about five minutes; the tubes containing the bloods were then corked and incubated. Ten bloods were tested in this way. The resistance of their corpuscles to hypotonic sodium chloride solution increased in all instances, but the results were somewhat variable, the increase being much more marked in some instances than in others. No evidence was obtained of the presence of an enzyme that affected the corpuscle so that an increase in fragility to hypotonic salt solutions resulted.

TABLE 7

Test of the effect on the resistance of human corpuscles of incubation in human euglobulin and beef euglobulin solutions and in a solution of sodium chloride after saturation with carbon dioxide

	HOURS INCUBATED	READING OF RESISTANCE TEST, PER CENT SODIUM CHLORID													
		0.03	0.06	0.09	0.12	0.15	0.18	0.21	0.24	0.27	0.30	0.33	0.36	0.39	0.42	0.45
Initial										4	3	1	0			
In human euglobulin	18					4	3	3	2	2	2	2	1	trace	trace	trace
Initial									4	3	3	2	1	trace	trace	0
In beef euglobulin	18									4	3	2	2	2	2	1
In beef euglobulin	48										4	3	2	1	1	1
Initial	0									4	2	1	trace	0		
In sodium chlorid with carbon dioxid	18	2	2	2	1	1	1	1	1	1	1	1	1	1		

The incubation of blood corpuscles with serum that had been treated with kaolin gave negative results so far as increase in fragility of the corpuscles was concerned. This was also true of treatment of corpuscles with serum that had been washed with chloroform.

The effect of injury to corpuscles on their rate of change of resistance. To see what effect slight degrees of injury of the corpuscles might have on

the rate of change of resistance, blood was heated in the water bath for one hour during which the temperature of the bath passed from 52 to 45°C. It was then incubated in isotonic sodium chloride solution. As compared with the normal control, the increase in resistance started sooner, but did not progress so far, the heated cell showing earlier signs of deterioration (table 8). Prolonged and rapid centrifuging also accelerated an increase in resistance. It would seem that a certain degree of injury of the corpuscles favors the rate of increase in its resistance.

TABLE 8

Test of the rate of change in resistance of corpuscles to hypotonic salt solution on incubation in isotonic sodium chloride solution after injury by heating

	DAYS INCUBATED	HEMOLYSIS IN SUPERNATANT FLUID	READING OF RESISTANCE TEST, PER CENT SODIUM CHLORID												
			0.08	0.11	0.15	0.18	0.22	0.25	0.29	0.33	0.36	0.40	0.43	0.47	0.51
Initial	0							4	3	2	1	1	1	0	
Normal cells	1	0						4	3	3	2	2	2	0	0
Normal cells	2	0	4	3	2	2	1	1	1	1	1	1	1	1	1
Initial	0							4	3	2	1	1	1	0	
Heated cells	1	+			4	3	3	2	2	2	2	1	1	1	1
Heated cells	2	+									4	3	2	2	2

SUMMARY

Human blood corpuscles have been incubated in commercial trypsin, steapsin, in 10 per cent egg albumen solution and in beef euglobulin.[2] When removed from these media and tested with a scale of hypotonic salt solutions they were found to have decreased in resistance.

No element was found in normal human blood which caused a decrease in the resistance of its corpuscles. Tests were made with the globulin

[2] Although I have been unable to find the original article, I have seen reference to some work by Pick and Silberstein upon the effect of treatment of corpuscles with various antisera, organ extracts, and foreign euglobulin. This work should be mentioned in this connection and I much regret that I am unable to be more direct in my quotation. Most of these reagents according to these authors produce upon incubation a decrease in resistance of the corpuscles to hypotonic salt solution; foreign euglobulin, however, caused an increase in resistance. It would seem probable that I have used a stronger solution of euglobulin than was used by Pick and Silberstein and have obtained evidence of the fragility producing effect of euglobulin before it was masked by the increase in resistance resulting from incubation in an excess of fluid, which was probably the effect noted by these authors. I have repeated my results with egg albumen and beef euglobulin and extended the incubation period. I find the same marked increase in fragility after two days incubation but a subsequent increase in resistance after four days' incubation.

fraction, the euglobulin fraction, with plasma treated with kaolin and with chloroform and plasma at an acid reaction.

Corpuscles incubated in an excess of fluid, whether the fluid was serum, a solution of the serum albumin fraction or a solution of either of the globulin fractions, or isotonic sodium chlorid solution, were subsequently found to have markedly increased in resistance to hypotonic salt solution with at the same time a certain amount of deterioration, as shown by the movement of the point of initial hemolysis to lower dilutions. Apparently the cell, after it has undergone a certain change, increases its resistance. This change is accelerated by injury, and to some extent by the serum fractions, less so by defibrinated and citrated plasma. A markedly rapid increase in resistance is brought about by saturation with carbon dioxid.

The increase in resistance with an increase in the range through which hemolysis takes place, which is found in the corpuscles of many patients with anemia has been reproduced in vitro in normal corpuscles. As the more resistant corpuscles in vitro are the result of deterioration, it would seem probable that, in any given specimen of blood, the more resistant cell is either an older or an injured cell.

BIBLIOGRAPHY

(1) Ashby: Journ. Exper. Med., 1921, xxxiv, 127.
(2) Bechold: München. med. Wochenschr., 1921, lxviii, 127.
(3) Brinkman: Demonstrated at the Eleventh International Congress of Physiologists, Edinborough, Scotland.
(4) Doisy and Eaton: Journ. Biol. Chem., 1921, xlvii, 377.
(5) Giffin and Sanford: Journ. Lab. and Clin. Med., 1919, iv, 465.
(6) Greenthal and O'Donnell: Amer. Journ. Dis. Child., 1921, xxii, 212.
(7) Hill: Arch. Int. Med., 1915, xiv, 809.
(8) Robertson: The physical chemistry of proteins. New York, 1918.
(9) Rohonyi: Kolloid Chem. Beiheft, 1916, viii, 391.
(10) Seyderhelm: Arch. f. Exper. Path. u. Pharm., 1918, lxxxii, 253.
(11) Wearn, Warren and Ames: Arch. Int. Med., 1922, xxix, 527.

A STUDY OF THE MECHANISM OF CHANGE IN RESISTANCE OF ERYTHROCYTES TO HYPOTONIC SALT SOLUTION

II. Quantitative Study of the Effect of Cations on Human Red Blood Corpuscles[1]

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In Study I, I recorded that by prolonged incubation of the human blood corpuscles in isotonic sodium chlorid solution, a marked increase in resistance to hypotonic solutions is produced. The purpose of the present study is to investigate the significance of the components of the sodium chlorid solution in this increase in resistance.

Under favorable conditions the increase in the resistance of corpuscles incubated for three or four days in isotonic sodium chlorid solution, is found, on subsequent testing with a series of hypotonic solutions, to be so great that the majority of the corpuscles will almost withstand water (table 1). The readings are on the basis of 0 to 4; 4 represents complete hemolysis, and "trace" a very slight amount. This corresponds with the degree of resistance to hypotonic sodium chlorid solution that I have found in white corpuscles, which, separated from the red corpuscles by differential centrifuging, and tested with hypotonic salt solution, will almost resist water, but are destroyed by pure water.

A similar degree of resistance to hypotonicity of beef corpuscles is reported by Bang (3) after incubating in sucrose solution, and in mixtures of sodium chlorid and a carbonate. He considers that the effect is due to decrease in osmotic pressure from loss of acid in the case of the sodium chlorid carbonate mixture, and of salts and acid in the case of the sugar solution. Sogen (11) finds that corpuscles kept in Rous' preserving solution, which is a mixture of isotonic sucrose, sodium citrate, and sodium chlorid, show an increase in resistance on subsequent test with a scale of hypotonic solutions. Gunn (5) increases resistance of corpuscles to hypotonic solutions by immersing them in a 1:10,000 solution of arsenous acid, and Aub (2) similarly produces an increased resistance with dilute solutions of lead acetate.

[1] Preliminary report given before the American Association of Immunologists, Washington, D. C., May, 1922.

In collaboration with the Clinical Section of Dr. H. Z. Giffin.

Since Hamberger first became interested in the problem of the permeability of the red blood corpuscle, a great deal of work has been done to determine the effect of various ions on the cell membrane. Höber (6) prepared slightly hypotonic solutions of the various alkali salts, and allowed blood corpuscles to stand in them until they hemolyzed, observing the order in which hemoglobin appeared in the supernatant fluid. He established the following series: Li < Na < CS < Rb < K for cations, and < SO_4 < Cl < Br < NO_3 for anions. He considers that the ions affect the membrane colloids differently, and in so doing alter the permeability of the cell membrane. This work of Höber's, however, or any other work in which the criterion for change in the cell is the appearance of hemoglobin in the medium, does not have a direct bearing on the data I am presenting, which was obtained by testing with a series of hypotonic solutions, the cells remaining after incubation. It is evident that the two methods are tests of two separate effects produced in the corpuscles, because the

TABLE 1

Increase in resistance of human corpuscles to hypotonic salt solution after incubation in isotonic sodium chloride solution

	READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID														
	0.03	0.06	0.09	0.12	0.15	0.18	0.21	0.24	0.27	0.30	0.33	0.36	0.39	0.42	0.45
Control test.....										4	3	2	1	1	0
Incubated four days.....	4	2	2	1	1	1	tr.*	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.

* tr. = trace.

results obtained by them do not run parallel. For instance, the incubation of human corpuscles in calcium chlorid will cause the release of large amounts of hemoglobin into the supernatant fluid, but the remaining corpuscles will have greatly increased in resistance to a series of hypotonic solutions; also, as I shall show later, corpuscles from dogs hemolyze to a much greater extent when incubated in potassium chlorid than when incubated in sodium chlorid, yet the remaining corpuscles, when tested with a series of hypotonic solutions, show an increase in resistance after incubation in potassium chlorid, and a decrease in resistance after treatment with sodium chlorid. Neuschlosz (10), using the same method as Höber, studied the antagonistic effect of sodium, potassium and calcium on one another when washed corpuscles from cattle were incubated in mixtures of their salts. He found that, in certain proportions, mixtures of the salts caused less hemolysis than any one salt. He relates these inhibiting effects to an effect on the surface tension of lecithin, and believes that the antagonistic effect of the salts on one another is due to an effect on the cell membrane colloids and their consequent resistance to osmotic

pressure. Although hemolysis by urethane, alcohol and saponin is not comparable to hemolysis by hypertonicity it is interesting to note that Miculicich, in studying the effect of cations on hemolysis by the first three agents, established the following series: K < Rb < Na < Ba < Ca < Mg < Sr. A tremendous amount of work has been done on the permeability and the mechanism of permeability of the corpuscle to various substances, from the researches of Hamberger and his students to those of Doisy and Eaton (4), Van Slyke and others.

THE EFFECT OF OSMOTIC PRESSURE. It was first necessary to rule out the possibility that the sodium chloride solution in which the corpuscles were incubated was hypotonic, and because of the hypotonicity permitted the corpuscles to decrease their osmotic pressure. In all of this work a 0.9 per cent solution of sodium chlorid (154/1000 M) is taken as the unit of comparison, this being assumed to be a representative isotonic solution. When solutions of other salts are used, they are of an equimolar strength,

TABLE 2

Increase in resistance of human corpuscles to hypotonic salt solution after incubation for two days in sodium chloride solutions of various percentages of isotonicity

SOLUTION	READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID												
	0.08	0.11	0.14	0.17	0.21	0.24	0.27	0.30	0.34	0.37	0.40	0.47	0.55
Initial resistance.....							4	3	2	1	trace	0	
In 200 per cent isotonic...		4	3	3	2	1	1	1	1	1	1	1	1
In 150 per cent isotonic...	4	3	2	1	1	1	1	1	1	1	1	1	1
In 100 per cent isotonic...	3	3	3	3	3	2	2	1	1	1	1	1	trace
In 80 per cent isotonic...	3	2	2	2	1	1	1	1	1	1	1	trace	trace
In 75 per cent isotonic...	3	3	3	3	2	2	1	1	1	1	1	trace	0

or a fraction thereof, depending on the valency of the component ions, and are referred to as isotonic solutions of their component salts. To test the effect of hypertonic and hypotonic solutions of salt on blood corpuscles, blood was mixed in 200 per cent (308/1000 M), 150 per cent (231/1000 M), 125 per cent (192/1000 M), 100 per cent (154/1000 M), 80 per cent (123/1000 M), and 75 per cent (115/1000 M) isotonic solutions of sodium chlorid and incubated at 36°C. for two days. At the end of this time the resistance of the corpuscles to a subsequent test with a series of hypotonic salt solutions had increased to about the same extent with each treatment. The hypotonic salt solutions used for testing the resistance were made of mixtures of 0.9 per cent sodium chloride solution and water: as the interval is cumbersome only two figures of the percentage are given. The readings are given in table 2. Apparently the osmotic pressure of the solution does not play a part in producing the increase in resistance.

THE EFFECT OF THE CATION. An attempt was made to learn whether the cation or the anion was responsible for the increased resistance. Isotonic solutions of sodium chlorid, potassium chlorid, sodium sulphate, potassium sulphate, sodium nitrate, and potassium nitrate were adjusted to pH 7.4 to 7.6, and were mixed with blood in the proportion of 1:10. The mixtures were incubated for two days with daily resuspension of the corpuscles, which is necessary to prevent too rapid deterioration. The results are given in table 3. It is evident that the anion plays a minor part.

The ability of the various cations to cause changes in resistance was investigated. Solutions of the chlorids of potassium, rubidium, cesium, lithium and sodium, which were molar equivalents of 0.9 per cent sodium chloride solution (154/1000 M) were made: similar solutions of the bivalent metals were used, except that two-thirds of a molar equivalent

TABLE 3

Test of the relative changes in resistance of human corpuscles to hypotonic salt solution after incubation in sodium salts and potassium salts

	READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID									
	0.17	0.21	0.24	0.27	0.30	0.34	0.37	0.40	0.47	0.53
Initial resistance					4	3	1	1	1	0
Two days in sodium sulphate	4	3	1	1	1	1	1	1	1	0
Two days in sodium nitrate		4	3	3	2	1	1	1	1	trace
Two days in sodium chlorid		4	3	3	2	1	1	1	1	trace
Two days in potassium sulphate								4	3	2
Two days in potassium nitrate							4	3	2	1
Two days in potassium chlorid							4	3	2	1

was taken. As it had been found that even large deviations from isotonicity did not materially alter the result, it was assumed that any differences in the degree of ionization of these salts at these concentrations would be of no consequence. The approximate equality of osmotic pressure was, however, verified by running resistance tests with the respective solutions against the same blood. The solutions were adjusted to approximately pH 7.5; mixtures in the proportion of 1–10 of blood and the various solutions were then made, placed in the incubator, and the corpuscles resuspended each day by rotating the container. After twenty-four hours there was no hemoglobin visible in the supernatant fluids of any of the mixtures. After three days there was a little reddening of the rubidium and potassium solutions in which the corpuscles were incubated, but no evidence of released hemoglobin in the lithium or sodium solutions. On testing with hypotonic salt solutions it was found that the corpuscles in rubidium and potassium solutions had decreased in resistance, while those

in lithium and sodium had increased in resistance. Cesium caused an increase in resistance comparable to that of lithium (table 4).

Blood corpuscles treated with solutions of the alkaline earth metals showed rapid and marked increase in resistance with, in the case of barium, strontium and calcium, much coincident hemolysis. Magnesium, however, caused no hemolysis after two days' incubation (table 5).

TABLE 4

The relative changes in resistance of human corpuscles to hypotonic salt solution after incubation in potassium, rubidium, lithium and sodium chlorids

	READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID													
	0.17	0.20	0.24	0.27	0.31	0.24	0.37	0.41	0.44	0.48	0.54	0.57	0.61	0.64
Initial resistance					4	3	2	1	trace	0				
One day in potassium chlorid					4	3	3	3	2	1	1	1	1	
Three days in potassium chlorid												4	2	1
One day in rubidium chlorid					4	3	3	2	2	1	1	1	1	
Three days in rubidium chlorid												4	3	2
One day in lithium chlorid				4	3	3	2	1	1	1	1	1	1	1
Three days in lithium chlorid			4	3	2	1	1	1	1	1	1	1	1	1
One day in sodium chlorid				4	3	3	2	1	1	1	trace	0	1	
Three days in sodium chlorid	4	2	2	1	1	1	1	1	1	1	1	1	trace	

TABLE 5

The increase in resistance of human corpuscles to hypotonic salt solution after incubation in isotonic magnesium, calcium, and strontium chlorid

	READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID													
	0.07	0.10	0.14	0.17	0.20	0.24	0.27	0.31	0.34	0.37	0.41	0.34	0.48	0.54	0.57
Initial resistance								4	3	2	1	1	trace	trace	0
Two days in magnesium chlorid		4	2	2	1	1	1	1	1	1	1	1	1	1	1
Two days in calcium chlorid	4	3	2	1	1	1	1	1	1	1	1	1	1	1	1
Two days in strontium chlorid	4	2	2	1	1	1	1	1	1	1	1	1	1	1	1

It will be seen that corpuscles from human beings become less resistant when incubated with salts of rubidum and potassium, and become more resistant when incubated with salts of lithium, sodium and the bivalent metals, barium, magnesium, strontium and calcium.

THE REVERSIBILITY OF THE CHANGES IN RESISTANCE. The reversibility of these reactions was tested. Blood was incubated with daily shaking in isotonic solutions of potassium, rubidium and sodium chlorids for three days, which produced the approximately maximal effect for each salt.

The respective corpuscles were then transferred to salt solutions producing the opposite effect. The results are given in table 6. The reaction is reversible and is more easily produced after the corpuscles have been incubated for a longer time, and presumably have deteriorated somewhat and become more permeable, which would indicate that this change in resistance is not attained through a modification of a membrane, but is concerned with that which is within the corpuscle. This increased ease in reversing the reaction after the corpuscles have changed to a greater extent with increased incubation is demonstrated in table 7.

TABLE 6

Test of reversibility of change in resistance of human corpuscles to hypotonic salt solution after incubation in isotonic sodium chloride solution and in isotonic potassium or rubidium chloride solutions

	READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID																	
	0.07	0.10	0.14	0.17	0.20	0.24	0.27	0.31	0.34	0.37	0.41	0.44	0.48	0.51	0.54	0.58	0.61	0.65
Initial resistance.....								4	3	2	1	tr.	0					
In potassium chlorid three days.....															4	2	1	1
Into sodium chlorid eighteen hours.....	4	2	1	1	1	1	1	1	1	1	tr.*	tr.	tr.					
Into potassium chlorid three hours.....					4	3	3	3	1	1								
In rubidium chlorid three days.....															4	3	2	1
Into sodium chlorid eighteen hours.....	4	1	1	1	1	1	1	1	1	1	1	1	tr.	tr.	tr.			
Into potassium chlorid three hours.....				4	3	3	1	1	1									
In sodium chlorid three days.....	4	3	1	1	1	1	1	1	1	1	1	1	1	1	1			
Into potassium chlorid eighteen hours.....						4	3	2	1	1	tr.	tr.						

* tr. = trace.

The increased resistance obtained by incubation with lithium and magnesium is like that obtained from incubation with sodium, reversible by means of potassium. In the tests made, the decreased resistance obtained by use of potassium increased again with sodium, showing that the decreased resistance was not merely a deterioration of the corpuscles. After treatment with calcium it was not possible to decrease the resistance with potassium, even after two days' incubation in potassium chlorid.

THE LIMITS OF EFFECTIVENESS OF THE CATIONS. Tests were made to see what degree of inhibition the oppositely acting salts had on one another.

To this end, mixtures of isotonic solutions of potassium and sodium chlorids and of rubidium and sodium chlorids were made in varying proportions,

TABLE 7

Test of the greater rate of reversal of the effect of incubation in potassium chlorid after an incubation of two days than after incubation of one day

	READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID										
	0.17	0.20	0.24	0.27	0.31	0.34	0.37	0.41	0.44	0.48	0.51
Initial resistance					4	2	2	1	0		
In potassium chlorid one day							4	3	3	2	1
Into sodium chlorid for forty minutes							4	3	2	1	1
Into sodium chlorid for five hours					4	3	3	3	3	2	2
Into sodium chlorid for one day	4	3	2	1	1	1	1	1	1	1	1
Into potassium chlorid two days									4	3	2
Into sodium chlorid for forty minutes			4	3	3	1	1	1	1	1	1

TABLE 8

Test of the balance of the effects of rubidium and potassium chlorids against sodium chlorid

CORPUSCLES INCUBATED FOR 2 DAYS IN MIXTURES OF		HEMOLYSIS IN MEDIUM AFTER INCUBATION	READING OF RESISTANCE TEST, PER CENT SODIUM CHLORID													
Parts of isotonic sodium chloride solution	Parts of isotonic potassium chloride solution		0.14	0.17	0.20	0.24	0.27	0.31	0.34	0.37	0.41	0.44	0.48	0.51	0.54	0.58
Initial resistance							4	3	2	1	0					
100	0	0	4	3	2	1	1	1	1	1	1	1	1	1	1	1
80	20	0		4	3	1	1	1	1	1	1	1	1	1	1	1
60	40	0				4	3	2	1	1	1	1	1	1	1	1
40	60	0						4	3	1	1	1	1	1	1	1
20	80	0									4	3	2	1	1	1
Parts of isotonic sodium chloride solution	Parts of isotonic rubidium chloride isotonic															
Initial resistance								4	3	2	1	0				
100	0	0			4	3	2	1	1	1	1	1	1	1	1	1
80	20	0					4	3	2	1	1	1	1	1	1	1
60	40	Faint trace						4	3	2	2	2	1	1	1	1
40	60	trace							4	3	3	2	2	1	1	1
20	80	trace										4	2	2	2	2

and incubated with blood for two days. The results are shown in table 8. Making allowance for the greater tendency of the corpuscles to deteriorate in rubidium than in potassium chlorid, it would appear in this

instance that an approximately 5 to 50 ratio was necessary before the point of complete hemolysis was moved beyond its initial place by either of the two metals that produce increased fragility, although a longer incubation period would have modified this result.

Blood was incubated for two days in mixtures of isotonic magnesium and potassium chlorids at approximately pH 7.5 with the results shown in table 9. As a result of incubation there was no released hemoglobin in the fluids of any of these mixtures. The effects of magnesium and potassium are approximately balanced in the proportion of 1–4.

To remove the influence of the second metal, and to see in what dilutions potassium alone was effective in bringing about a decrease in resistance, mixtures of isotonic sucrose and potassium chlorid were made. These were brought to approximately pH 7.5, mixed with blood in the proportion of 1–10, and incubated for three days (table 10). These mixtures

TABLE 9

Test of balance between the effects of magnesium chlorid and potassium chlorid

CORPUSCLES INCUBATED IN MIXTURES OF		READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID														
Parts of isotonic magnesium chloride solution	Parts of isotonic potassium chloride solution	0.06	0.09	0.12	0.15	0.18	0.21	0.24	0.27	0.30	0.33	0.36	0.39	0.42	0.45	0.48
Initial resistance									4	3	2	1	1	trace	trace	0
1	0	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1						4	3	3	2	2	1	1	1	1	1
1	4								4	3	3	2	1	1	1	1
1	10									4	3	3	3	2	1	1
1	20									4	3	3	2	1	1	1
0	1									4	3	3	3	3	1	1

were tested every day and cultured to test sterility. On the second day the corpuscles in the 40 to 60 mixture gave a higher resistance than they did on the third day. On the third day they were found to be contaminated. The third day's reading, however, has been given. From these results it appears that potassium chlorid must be in a concentration of at least 80 per cent isotonic solution, to decrease resistance. This is the same ratio found when it was mixed with magnesium. As the potassium content of normal corpuscles, as given by Kramer and Tisdall (7), is equivalent to the potassium content of about 70 per cent isotonic potassium chlorid solution, it would seem that in order to maintain a resistance equilibrium in the presence of sugar or magnesium, it is necessary to have as much potassium outside the corpuscle as within. As the relationship of potassium to sodium in the plasma is something less than a 1–10 ratio, the fact that in the test tube a ratio of more than 1–1 of potas-

sium to sodium is necessary for a decrease in resistance would suggest that the adult corpuscle is not decreased in resistance by physiologic increases in potassium in the blood stream.

TABLE 10

Test of balance between the effects of isotonic potassium chloride and isotonic cane sugar solutions

CORPUSCLES INCUBATED IN MIXTURE OF		COLOR OF MEDIUM AFTER INCUBATION	READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID																	
Parts of isotonic sugar solution	Parts of isotonic potassium chloride solution		0.03	0.07	0.10	0.14	0.17	0.20	0.24	0.27	0.31	0.34	0.37	0.41	0.44	0.48	0.51	0.54	0.58
Initial resistance									4	3	3	2	1	1	0				
100	0	Red	4	2	2	1	1	1	1	1	1	1	1	1	1	1	1		
80	20	Pink	4	3	2		2	2	1	1	tr.*	tr.	tr.	tr.	tr.				
60	40	Straw		4	3	2	2	2		1		tr.	tr.	tr.	tr.				
40	60	Straw					4	3	3		2		1	1	1		tr.		
30	70	Straw						4	3		2		1	1	1	1	1		
20	80	Straw							4	3	2	1	1	1	1	1	1		
10	90										4	3	2	2	2	2	2		
0	100	Pink															4	3	2

(To see that this increase in fragility in the 100 per cent potassium was not due to injury, these corpusles were transferred to a sugar solution with the following results)

100	0		4	3	2	1	1	1	1	1	1	1	1	1	1	1	1		

*tr = trace.

TABLE 11

Test of balance between the effects of potassium and calcium chloride solution

CORPUSCLES INCUBATED IN MIXTURES OF		DAYS INCUBATED	COLOR OF MEDIUM AFTER INCUBATION	READING OF RESISTANCE TEST, PER CENT OF SODIUM CHLORID							
Parts of isotonic calcium chloride solution	Parts of isotonic potassium chloride solution			0.24	0.27	0.30	0.33	0.39	0.42	0.45	0.48
Initial resistance				4	3	3	2	1	trace	trace	0
1	4	2	Red		4	3	3	2	2	1	trace
1	7	2	Red		4	2	2	2	1	1	trace
1	10	2	Pink	4	3	2	1	1	1	1	trace

Calcium, whose action was found to be not reversible, behaves differently from the foregoing salts. It causes increase in resistance in very low dilutions. When mixed with sodium, it accelerates the increase in resistance which takes place in the presence of that metal; when mixed with

potassium, it causes destruction of the corpuscles when it is present in larger amounts, and may cause an increase in resistance in spite of the potassium present when it is mixed with the potassium in smaller amounts. Table 11 illustrates the result of incubating corpuscles in mixtures of isotonic calcium and potassium chloride solutions for two days. It is evident that this apparent increase in fragility is in reality a cell injury because, when transferred to sodium chlorid, only the corpuscles in the 1–10 mixture which showed a slight increase in resistance continued to increase in resistance.

A further study of the anions showed that, as a rule, when they did not injure the cell and cause a deterioration that masked the relative effect of sodium and potassium (which was tested by reversing the reaction), the resistance increased in a sodium salt and decreased in a potassium salt. Bromids, iodids, nitrates, oxylates, phosphates and acetates were tested.

The citrates proved to be exceptions. In both sodium and potassium citrate, corpuscles increased in resistance to the same extent that they did in sugar.

Normality of the treated corpuscles. Corpuscles whose resistance is changed by treatment with potassium or sodium chlorid stain normally with Wright stain and with brilliant crystal blue. Pathologic blood that initially contained 12.6 per cent reticulated corpuscles, after three days' incubation in sodium chlorid solution, with increase in resistance, gave a count of 13.0 per cent of well-staining reticulated red cells; this was rather surprising, as in the blood stream a wave of increase in reticulated cells will sometimes not last more than two days. Corpuscles incubated in calcium on the whole stained normally with Wright stain, but showed a considerable proportion that were of a copper color, like that seen in erythroblasts in the circulation. Group II corpuscles (Moss) which had decreased in resistance by treatment with a potassium salt, or increased in resistance with treatment by a sodium salt, were just as agglutinable when treated with group IV serum as the untreated corpuscles after two days' incubation. With injury of the corpuscles, as indicated by hemolysis, they become less agglutinable. Corpuscles treated with calcium almost entirely lost their agglutinability. Aub's statement that lead-treated corpuscles lose their agglutinability suggested the foregoing test. Tests were not made on animals to see if their corpuscles would support life after treatment with these various salt solutions, as it did not seem that the data which could thus be attained would warrant the work entailed in securing them. The decreased resistance produced by incubation in potassium and rubidium, for instance, is due to a change in the corpuscles which is related to their resistance, and not to general deterioration, because their resistance to hypotonic sodium chlorid solution can be restored to them by replacing the potassium with sodium. As to whether

or not the corpuscles by this time have undergone a general deterioration which would unfit them for life in the circulation is an open question and not directly connected with the problem.

SUMMARY AND CONCLUSIONS

The resistance of the red blood corpuscles of human beings decreased on incubation in isotonic rubidium or potassium chlorid at approximately pH 7.4. This association of rubidium and potassium is in line with their association in connection with other biologic phenomena, such as the similarity of their pharmacologic action and, as Loeb (8) has shown, their ability to replace one another in balancing otherwise toxic salt solutions. The resistance of such corpuscles to hemolysis by hypotonic sodium chloride solution can be increased by incubation in lithium, sodium and magnesium chlorid without cell injury, as evidenced by the appearance of hemoglobin in the medium. Resistance is greatly increased in calcium, strontium and barium chlorids with much cell injury, as indicated by coincident hemolysis.

The decreased resistance obtained by incubation in solutions of salts of potassium or rubidium can be reversed by replacing these metals with any of the second group, and so does not depend directly on injury of the cell, or loss or destruction of the cell substance.

Increase in resistance obtained by incubation in lithium, sodium or magnesium chlorids, is reversible in potassium and rubidium chlorids, and therefore does not depend on any coagulation or precipitation of the cell substance. The increase in resistance produced by calcium is not reversible.

It would seem that the change in resistance to hypotonic salt solution resulting from treatment with the salts of metals whose effect can be reversed, is not the direct result of a change in the cell membrane, because the further the change has progressed, the more easily the resistance can be reversed.

From the data obtained by the study of mixtures of the various metal ions it seems possible that potassium is an active agent in causing decrease in resistance, and that the increase in resistance which results when magnesium or sodium is substituted for it, may be caused by the loss of potassium. This is assumed because magnesium chlorid behaved like sucrose, balancing the potassium effect when the concentration of potassium was approximately the same outside the cell as within.

As the decrease in resistance produced by incubation in potassium and rubidium chlorids, and the increase in resistance produced by incubation in lithium, sodium and magnesium chlorids, are reversible, it is evident that they are independent of any loss of anions from the corpuscle. The resistance of corpuscles which is increased on incubation

in isotonic sodium chlorid, for instance, is decreased again when the potassium or rubidium ion is substituted for the sodium ion. As the cation, only, has been changed, the reversal in resistance must depend on the change of cation. It becomes evident that the cation content of the red blood corpuscles must be one factor in determining their resistance to hypotonic sodium chloride solution.

BIBLIOGRAPHY

(1) Ashby: This Journal, 1924, lxviii, 239.
(2) Aub and Smith: Journ. Amer. Med. Assoc., 1922, lxxviii, 1754.
(3) Bang: Biochem. Zeitschr., 1909, xvi, 255.
(4) Doisy and Eaton: Journ. Biol. Chem., 1921, xlvii, 377.
(5) Gunn: Brit. Med. Journ., 1908, ii, 145.
(6) Höber: Biochem. Zeitschr., 1908, xiv, 209.
(7) Kremer and Tisdall: Journ. Biol. Chem., 1922, liii, 241.
(8) Loeb: Journ. Gen. Physiol., 1921, iii, 237.
(9) Miculicich: Zentralbl. f. Physiol., 1910, xxiv, 523.
(10) Neuschlosz: Arch. f. d. gesammt. Physiol., 1920, clxxxi, 40.
(11) Sogen: Tokuhu Journ. Exper. Med., 1920, i, 367.

THE PROCESS OF PHOTIC ORIENTATION IN THE ROBBER-FLY, PROCTACANTHUS PHILADELPHICUS[1]

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The idea that orientation in bilaterally symmetrical animals is the result of antagonistic or balanced action in the locomotor appendages on opposite sides, and that this action is specifically controlled by the relation in the amount of stimulating energy received by the receptors on opposite sides, is widely accepted. This idea originated in Ray's explanation of orientation in plants (1693).

Ray maintained that plants in a window turn toward the light because, owing to differences in temperature, growth is more rapid on the room-side than it is on the window-side. According to this explanation orientation is dependent upon the relative activity of the motor mechanism on opposite sides of the reacting organ and this is specifically related to the relation in the amount of energy received by these two sides. DeCandolle (1832) followed Ray in principle, merely substituting light for temperature as the stimulating agent. Verworn (1894) accepted this "antagonistic-action" hypothesis and applied it to the lower animals. Loeb had at this time applied it in part to animals. He maintained in 1893 that orientation in insects and some other related organisms is dependent upon the antagonistic action of the locomotor appendages on opposite sides, but he held with Sachs (1876)[2] in opposition to DeCandolle, that the activity of these appendages is controlled by the direction in which the rays penetrate the tissue and *not by the amount of stimulating energy received by the receptors on opposite sides.*[3] This view he still advocated in 1903 (1905, p. 2). In 1906, however, he appears to have abandoned

[1] I am much indebted to Prof. J. S. Hine for identification of the specimens used, and to the Marine Biological Laboratory, Woods Hole, for experimental facilities.

[2] Sachs presented his views in an introduction to a series of papers by Müller (1876).

[3] This is very clearly stated in an earlier paper (1888, p. 2.) as follows: "Die Orientirung der Thiere gegen eine Lichtquelle wird wie bei den Pflanzen (J. v. Sachs) bedingt durch die Richtung in welcher die Lichtstrahlen die thierischen Gewebe durchsetzen, und nicht durch die Unterschiede in der Lichtintensität auf den verschiedenen Seiten des Thieres."

it in favor of the Ray-Verworn concept[4] which he still holds (1918). Among those who have accepted in full the Ray antagonistic action hypothesis as applied to animals by Verworn, Loeb and others, Bohn has given the most lucid exposition. He says, (1909 a, p. 9): "The side which is more strongly stimulated by the light will move more rapidly than the other (marchera plus vite que l' autre), and this results in a forced turning movement." And again (1909 b, p. 4) "Orientation (tropisme) presents itself to us as the forced result of the inequality of the *same* activities in the right and the left half of the body, an inequality which is purely quantitative, not qualitative." "In other words, orientation (tropisme) is not an activity (une activité), it is the result of an inequality between certain activities, simple or complex, which occur on one side of the body and the same activities which occur on the opposite side."

Some of the advocates of the Ray-Verworn hypothesis maintain that the antagonistic action is controlled by the effect of the stimulating agent on muscle-tonus (Loeb, 1893 and 1897; Bohn, 1904; Garrey, 1918; Minnich, 1919). According to the explanation of orientation by these investigators, the essential factors involved in the process may be formulated as follows:

1. Orientation is dependent upon the relation in the activity of the locomotor appendages on opposite sides, the right and the left appendages working independently in an antagonistic fashion. 2. The difference in the activity of these appendages is due to difference in muscle-tonus, it is purely quantitative, not qualitative. 3. This difference is due to difference in the amount of stimulating energy received by the receptors on opposite sides. 4. The activity of the appendages on one side is controlled entirely by impulses received from the receptors on one side in accord with the mechanism involved in the process of orientation in Hammond's mechanical dog (Loeb, 1918).

Garrey (1918) maintains that the results obtained in observations on Proctacanthus prove that the Ray-Verworn theory is correct. He found in Proctacanthus, just as Holmes (1905) had found in Ranatra, and others in certain other insects, that if one eye is covered the insect tilts strongly and turns continuously toward the functional eye. He contends that the tilting and the turning are both due to difference in the tonus of the muscles of the legs on opposite sides, resulting in extension in those on the one side and flexure in those on the other, that the difference in tonus is caused by difference in the amount of light received by the two eyes and that the degrees of tilting and turning are directly proportional to this difference. I have repeated and extended Garrey's observations.

[4] Often designated the "tropism" theory, the "Muscle-tension" theory, "Loeb's muscle-tension" theory, the "continuous action" theory, the "difference of intensity" theory, etc.

The results obtained lead to conclusions in reference to the process of orientation which differ considerably from those reached by Garrey. They strongly support the conclusions reached in observations on Eristalis and Erax (Mast, 1923), namely, that orientation in insects is the result of series of reflexes dependent upon localization of the stimulus in the eye, and that the Ray-Verworn theory or any other theory that rests upon the idea that orientation is necessarily dependent upon balanced or antagonistic action in the receptors and the locomotor appendages on opposite sides, is inadequate to account for the observed facts.

MATERIAL AND METHODS. The robber-flies are usually found in dry, warm places well exposed to the sun. They feed on other insects and have the habit of sitting perfectly motionless for considerable periods of time waiting for prey. Many of their responses are associated with this habit.

Proctacanthus does not thrive in captivity where it rarely if ever feeds. It is consequently necessary to make frequent collections in order to have specimens in prime condition continuously at hand for experiments and observations. This offered no serious difficulties at Woods Hole, where the following work was done, for although Proctacanthus was not abundant, it was always possible to obtain sufficient material.

The observations were all made in a dark room, either in natural light in front of a window or in artificial light in front of a lamp in a light-tight box containing an opening of such a nature as to produce a well-defined horizontal beam.

Asphalt varnish was used to cover the eyes in all observations on totally or partially blinded specimens. Considerable difficulty was encountered in getting the asphalt evenly spread and hardened in sufficient quantities to exclude all light and in covering precisely limited areas. This difficulty was overcome by cutting off the tip of a pipet-bulb and pushing the specimens into the hole at one end just far enough so that the head projected from that at the other end. By this means the legs were securely held so that the insect could be placed in any position desired for covering the eyes and it could be held until the varnish was dry. In covering restricted regions of the eyes the varnish was always applied under the binocular.

The robber-flies are not agile on foot. On smooth hard surfaces they have great difficulty in walking at all. In making the following observations they were placed on fairly coarse paper. On this they can walk fairly well, but at best they are rather inactive. They frequently stop in the midst of an experiment and remain motionless indefinitely. However, if the abdomen is lightly touched when they stop, they usually continue on their course without any observable change in direction.

The courses taken were recorded by following the specimens with a pencil.

OBSERVATIONS AND RESULTS: *Specimens with both eyes covered.* Garrey (1918) maintains that robber-flies and butterflies with both eyes covered are sluggish and move about in a most incoördinate way, with the legs "abnormally extended and the proboscis and ventral aspect of the thorax in contact with the surface, as if the body weight could not be supported." He holds that this "points to a decreased neuromuscular tonus which is normally maintained reflexly by the action of light on the eyes" and that it supports the tonus hypothesis of orientation.

I made detailed observations on a number of different specimens with both eyes covered. In each of these specimens the posture during rest and locomotion (elevation of the body, spread of the legs, etc.) was carefully noted before the eyes were covered and again shortly after they had been covered. No consistent difference was observed.[5] Under both conditions the thorax during locomotion was held well above the surface, frequently as much as 5 mm. After walking a short distance which varied greatly (10± cm.) the specimens under both conditions came to rest and usually settled down until the ventral surface of the thorax rested on the table. Similar results were obtained in observations on active and inactive normal individuals exposed in light which differed in luminous intensity from direct sunlight at noon to light so weak that the specimens could barely be seen. In the weakest light the specimens appeared to walk just as readily and rapidly as in the strongest and no difference was observed in the spread of the legs or the elevation of the head, thorax or abdomen. In one specimen the spread of the middle legs was measured in three different intensities. The readings were made successively in the three intensities beginning with the lowest, and three readings were made in each. In each test the specimen was allowed to move toward the light until it came to rest, after which the position of the two middle feet was marked and the distance between them recorded. The following results were obtained: Lowest intensity, 33, 34, 32.5 mm.; average 33.16 mm. Medium intensity, 33.5, 32, 32.5 mm.; average 32.66 mm. Highest intensity, 34, 31, 32.5 mm.; average 32.83 mm.

These results seem to prove that if light has any immediate effect on neuromuscular tonus in Proctacanthus, it is very slight. They have no bearing on the effect of long exposure in different intensities and it is entirely possible that the disagreement between the results obtained by Garrey and myself is due to difference in the time of exposure. However this may be, it is clear that if light affects muscle-tonus only after long

[5] Results somewhat similar to these have also been observed in other arthropods. Radl (1903) found that, while flies cannot walk immediately after they are blinded, they walk very well if they are kept for some time. Parker's (1903) observations indicate that blind Vanessa has no serious difficulty in flying; and Cowles (1908) says that blind Ocypoda moves about readily and feeds.

exposure, such effects can have no profound bearing on orientation, for the orientation responses occur immediately after the illumination is changed.

Reactions in normal specimens: If Proctacanthus is laterally illuminated it turns directly toward the light and proceeds in a fairly straight course toward the source, i.e., it orients directly and moderately precisely. The sharpness of the turn toward the light depends upon the position of the source in relation to the longitudinal axis of the insect. If the source of light is only slightly to one side of a point directly in front of the insect, it turns very gradually. The farther to the side and to the rear the source of light is, the sharper the turn will be until the source of light is directly opposite the posterior edge of the eye, when the turning is so sharp that all forward motion ceases, and if the light is moved so as continuously to hold this position in relation to the eye, the insect turns round and round without locomotion, all of the legs on one side stepping continuously forward and all on the other continuously backward. In this reaction the feet do not, however, move directly forward or backward. The two front feet deflect sharply sidewise toward the light while the two hind feet deflect in the opposite direction. This seems to indicate clearly that orientation is not necessarily the result of a quantitative difference in the activity of the locomotor appendages on opposite sides, e.g., difference in the rate of movement; but that it is brought about by a qualitative difference, the feet on one side moving forward, with certain lateral deflections and those on the other side backward, with radically different lateral deflections.

Garrey maintains, as previously stated, that orientation is due to tilting which in turn is due to difference in the degree of extension of the legs on opposite sides owing to difference in muscle-tonus caused by difference in the amount of light received by the two eyes.

I made numerous detailed observations in reference to the posture of normal specimens of Proctacanthus during the process of turning toward the source of light. Under certain conditions it was evident that the insects lean toward the more highly illuminated side, the side toward which they turn in accord with Garrey's postulate, but under others there was no observable leaning whatever. It consequently appears that leaning or tilting is not essential in the process of orientation. This contention is strongly supported by results obtained in the following observations on insects with portions of the eyes covered.

Specimens with portions of the eyes covered: If Proctacanthus with one eye covered is placed on a white background (filter paper) in front of a window, it leans strongly and turns sharply toward the functional eye. It usually comes to rest, however, after moving a short distance; but if it is mechanically stimulated every time it comes to rest it continues to deflect toward the functional eye, making circus-movements. The extent of lean-

ing and the rate of turning are, under these conditions, dependent upon the intensity of the illumination. In direct sunlight both are greater than in diffuse daylight. Very little if any difference was, however, observed in different intensities of diffuse daylight. The relation between intensity and the extent of leaning is apparently not proportional.

On a white background in a given illumination the insects lean over much farther and turn more sharply than on a black background in the same or even a much higher illumination. This is very evident in specimens that are allowed to walk toward the window from a white onto a black piece of paper. As they pass from the one on which they may be leaning 30° or more to the other, they suddenly straighten up almost completely. The angle of leaning on white and black backgrounds was measured in a number of cases. A typical series of results follow: White background 17°, 28°, 22°, 30°, 30°. Black background 2°, 3−°, 2−°.

Similar results were obtained in specimens in which the lower half of one eye and the upper half of the other was covered. These results seem to demonstrate conclusively that tilting is not primarily dependent upon difference in the amount of light received by the two eyes, but upon the localization of the stimulus in the eye. This conclusion is strongly supported by the fact that if the ventral surface of one eye is illuminated and that of the other is not, the insect leans toward the former even if the latter receives much more light. This can be readily demonstrated by covering the lower half of one eye and the upper half of the other. Specimens treated thus lean toward the eye with the ventral surface uncovered. This eye receives light which is reflected from the background while the other eye receives direct light, which is, of course, ordinarily very much more intense.

Moreover, if in specimens with one eye entirely covered, the upper half of the other eye is also covered, the extent of leaning will be increased, although the difference in the amount of light received by the two eyes has been reduced.

Furthermore, under certain conditions the insects lean toward the eye which receives least light even if this eye is entirely covered, as the following observations show: On September 15 the right eye of a specimen collected the preceding day was thoroughly covered. As soon as the asphalt was dry, the insect was exposed in a well defined beam of light in the dark room, so arranged that the black background received practically no light. It leaned definitely to the right, the blind side, and turned in this direction. The leaning was particularly marked when the specimen turned so that the blind side faced the light. In this position the dorsal surface of the left eye was illuminated and the leaning toward the blind side was doubtless due to the localization of the stimulus in this region (fig. 1, A). In diffuse daylight this specimen consistently leaned and turned toward the functional

eye. It is consequently evident that tilting is not specifically related to difference in the amount of light received by the two eyes.

After a specimen with one eye covered has assumed a posture, say *e.g.*, on a white background, on which as previously stated it leans much more than on a black background, it retains this posture for considerable periods of time no matter how much the illumination may be changed. This was repeatedly observed. Details in reference to one series of observations follow: On September 12, 9 a.m., a specimen with the right eye covered was placed on a white background in strong diffuse daylight. It soon came to rest with the left front foot drawn in well toward the middle of the body and leaning over 23°. The window shutter was now repeatedly alter-

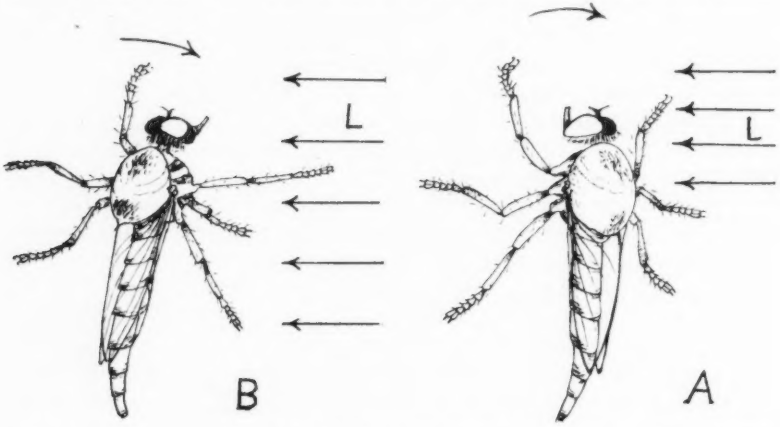

Fig. 1. A. Proctacanthus on a black background in a well-defined horizontal beam of light, *L*; right eye covered. Note that it is leaning toward the covered eye and that the upper median surface of the functional eye is directed toward the light. B. Proctacanthus on a white background in front of a window; the upper portion of the left eye and the lower portion of the right eye covered. Note that it is leaning to the left and turning to the right toward the light, *L*.

nately closed and opened, reducing the illumination until the insect was barely visible and increasing it to strong diffuse daylight. Moreover, this illumination was in some instances greatly augmented by reflections from white sheets of paper and mirrors. The insect was for 15 minutes very closely observed while these changes in illumination occurred. Not a movement of any kind except those connected with respiration was seen. At the close of this period, while the illumination was very low, the insect was touched with a needle. It immediately straightened up almost completely; then it became motionless again with one front foot raised about 2 mm. from the table. It was now carefully observed for 5 minutes while the intensity of the light was repeatedly changed as during the

preceding period. Again there was no observable movement until, in response to mechanical stimulation in strong illumination, it again immediately leaned over about 23°.

These facts demonstrate conclusively that whatever it may be that causes the assumption of the leaning posture, its retention is certainly not dependent upon the continuous action of the light. I am inclined to think that the assumption of the leaning posture is far more likely to be the result of something in the nature of a reflex response or series of responses than the result of tonus-effects and that the retention of this posture is the result of a muscular state like that which is assumed in death-feigning, a state which certainly stimulates what is ordinarily thought of as a tonus-effect. These two types of muscular phenomena have, however, not been clearly differentiated. The outstanding perceptual difference apparently consists chiefly of a time element, muscular contraction owing to increase in tonus being much more permanent than muscular contraction in reflex responses. It is probable that there are factors involved in these two types of phenomena which differ fundamentally. Jordan (1922) has recently made some interesting suggestions regarding this matter.

As previously stated, Proctacanthus usually turns in the direction in which it leans. This fact led Garrey to postulate an intimate relation between the two phenomena. As will be recalled, he maintains that both are the result of difference in muscle-tonus in the legs on opposite sides caused by difference in the amount of light received by the two eyes. These two phenomena do not, however, appear to be inseparably related, for specimens leaning strongly in one direction were repeatedly observed to take a straight course, to go sidewise toward the light in the opposite direction or actually to turn toward the blinded side, i.e., in a direction precisely opposite that demanded by Garrey's formulation.

These reactions are especially prominent in specimens with the lower portion of one eye and the upper portion of the other covered. If such specimens are exposed on a white background in front of a window, with the side containing the eye which has the lower portion covered facing the light, they usually, while leaning strongly from the window, go either sidewise toward the window, or straight ahead, or they turn toward the window (fig. 1, B). When they go sidewise toward the light all of the legs step sidewise in the same direction. When they turn toward the light the two front legs step sidewise more extensively than the others. Under these conditions the functional portion of the eye on the shaded side of the animal is directed downward and receives light largely if not entirely reflected from the white background, while the functional portion of the eye on the illuminated side is directed upward and receives light directly from the window. Since the organism leans toward the former and turns toward the latter, it seems evident that the leaning is due to the localiza-

tion of the stimulus in the lower portion of one eye and the turning to the localization of the stimulus in the dorso-lateral portions of the other eye. The former results in flexure of the legs on one side and extension in those on the opposite side, the latter in sidewise stepping toward the light, especially in the front legs. There consequently seem to be two distinct sets of responses, in both of which all of the legs are involved at the same time; and both of these sets of responses appear to be the result of an attempt to turn toward the region of the eye stimulated, the ventral in one and the dorso-lateral in the other. These results, as will be shown presently, strongly support the idea that localization and integration of stimuli are essential factors in orientation and they make is perfectly evident that turning is not necessarily causally associated with leaning or tilting.

Circus-movements: Turning may result either in orientation or in circus-movement. Consequently if the conclusion that turning is not necessarily associated with leaning or difference in flexure or extension in the legs on opposite sides is valid, it is evident that neither orientation nor circus-movement is necessarily associated with it.

Circus-movement as is well known occurs in many different organisms. Photopositive organisms in which the receptors on one side have been functionally destroyed usually turn, in non-directive light, continuously toward the normal side, taking roughly circular courses. It is this response which constitutes the main support of most of those who hold the Ray-Verworn theory. These investigators maintain that circus-movements in insects with one eye covered can be explained only on the assumption that the feet on one side move faster than those on the other side, that this difference in activity is due to difference in tonus in the muscles of the legs on opposite sides, resulting in extension in the one and flexure in the other, and that this difference is due to difference in the amount of light received by the two eyes, owing to the continuous action of the light on the tonus of the muscle in proportion to the amount received by the receptor connected with it. They maintain, moreover, that the factors involved in orientation are the same as those involved in circus-movements. Are these contentions valid?

The fact that under certain conditions some insects with one eye covered, e.g., Vanessa, go round and round in circles with the legs on one side strongly extended and those on the other strongly flexed and the fact that under certain conditions, the size of the circles becomes larger or smaller in accord with decrease or increase in the luminous intensity of the field, seem to support the view presented above. There are, however, certain features in connection with the reactions of such organisms which indicate that these views must be profoundly modified.

It has been observed in a considerable number of different species by Radl (1903), Holmes (1905), Carpenter (1908) Dolley (1916), Minnich

(1919), and others that orientation is still possible in specimens with one eye blinded. In these it is perfectly evident, as has long since been pointed out (Mast, 1911, p. 216) that some if not all of the factors mentioned above are not essential in the process of orientation. The photoreceptors on opposite sides certainly do not receive the same amount of light when the organism is oriented. But it is maintained (Minnich, 1919) that these organisms with one eye covered orient only after experience and the lapse of time showing that readjustment is necessary, and that orientation in them consequently has no bearing on the process of orientation in normal individuals. Are experience and time for readjustment actually necessary for orientation in one-eyed insects?

If Proctacanthus with one eye covered is exposed on a white background in diffuse light in front of a window it makes circus-movements toward the functional eye as previously stated. But if one eye and the lower or the postero-lateral portion of the other eye are covered it at once orients practically as precisely as it does with both eyes uncovered. This is clearly demonstrated in the reproduction of tracings of series of paths made by specimens, *1*, with both eyes normal; *2*, with one eye covered; and *3*, with one eye and portions of the other covered (figs. 2 and 3). In all of these experiments the individuals were firmly held in rubber bulbs during the entire time between the various tests. They consequently received no motor experience and the time that elapsed was only a few moments, for the tests were made in every case as soon as the asphalt applied was dry enough to hold. By referring to these figures it will be seen that both of the individuals represented oriented fairly precisely in the preliminary tests made with both eyes normal, that with one eye covered they deflected sharply toward the functional eye making complete circus-movements, that they deflected less and less as more and more of the other eye was covered until they oriented practically as precisely as they had oriented in the control tests. Similar results were also obtained in observations on Eristalis. It is consequently evident that insects which receive light only in one eye can, without experience or time for readjustment, orient practically as precisely as they do when both eyes are normal, provided the light received is localized in the anterior portion of the eye, and if this is true it must be concluded that orientation in normal specimens is not necessarily dependent upon the relation in the amount of light received by the two eyes. This contention is further supported by the results obtained in observations on the reactions of insects in light from two sources.

Orientation in light from two sources: If Proctacanthus is exposed in light from two sources it orients and travels toward a point between them. The location of this point depends upon the relation in the amount of light received from the sources. If it is equal and the sources are of the same size and at equal distances the point is approximately half way between

them. If more light is received from one source than from the other and they are the same in other respects, it is nearer the former than the latter. This holds for a considerable number of different organisms (Mast, 1911).

When an insect is proceeding toward a point half way between two sources of light of equal intensity and size and at equal distances it is evident that the two eyes are equally illuminated in all respects, provided the

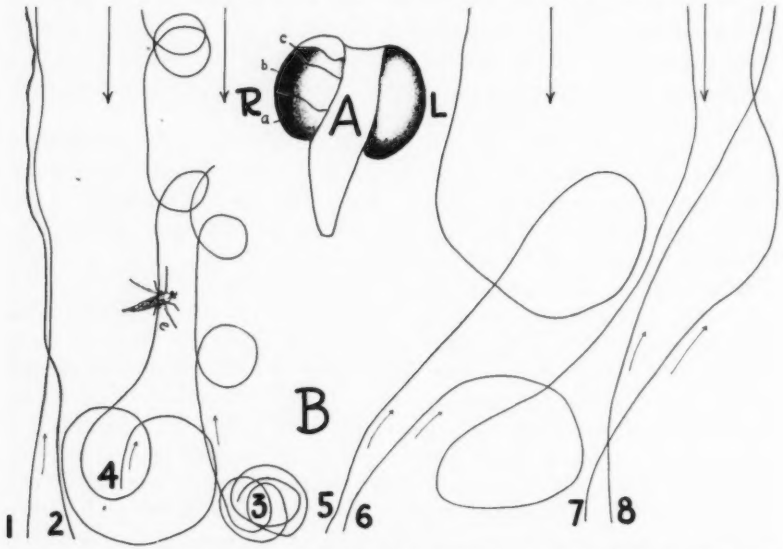

Fig. 2. A. Camera outline of face of Proctacanthus as seen from in front and slightly to one side; *R*, right eye; *L*, left eye; *a*, *b* and *c*, portions of right eye successively covered with asphalt varnish. B. Tracings of paths made in front of window by the individual represented in A; *1* and *2*, individual normals; *3* and *4*, left eye covered; *5* and *6*, left eye and *a* of right eye covered; *7* and *8*, left eye and (*a* and *b*) of right eye covered; large arrow, general direction of light from window; small arrows, direction of locomotion; *e*, sketch of individual leaning to the right and going sidewise to the left toward the light. Note that the insect went more and more nearly directly toward the light as more of the ventral and lateral surfaces of the right eye was covered until it went practically as directly toward the light as it did with both eyes uncovered.

two lights and the insect are continually on the same horizontal plane, but that they are not equally illuminated when the insect is exposed to lights of different intensities and goes toward a point nearer the stronger one. Under such conditions one eye receives more light than the other when the insect is oriented, the stimulus in the one is located farther forward than that in the other and the greater the difference in the intensity of the two lights the greater the difference in the location of the stimuli.

This matter was fully discussed in a former paper referring particularly to Eristalis (Mast, 1923, p. 151). It brings out in a striking way the untenability of the contention that when insects are oriented the receptors on opposite sides must be equally illuminated.

Reaction in specimens with some of the legs removed: Turning in Proctacanthus is largely dependent upon the action of the front legs. Consequently if orientation is the result of balanced, relative or antagonistic action of the locomotor appendages on opposite sides, specimens ought to

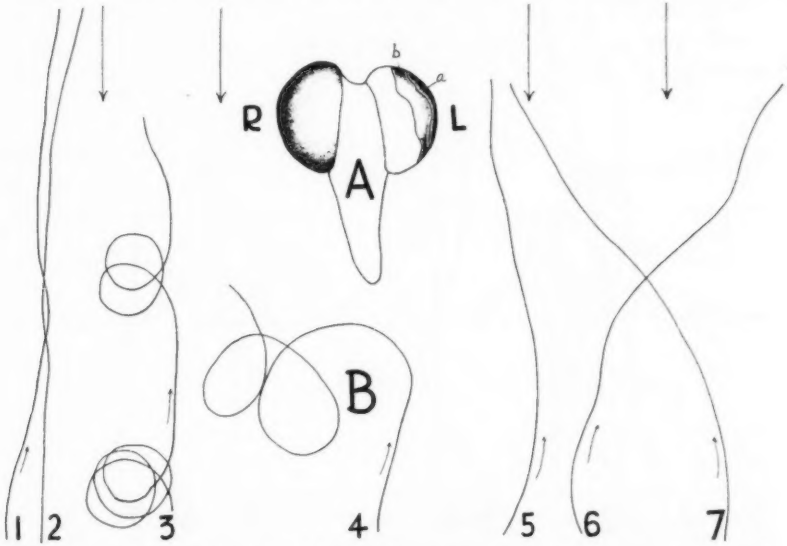

Fig. 3. A. Camera outline of face of Proctacanthus. *R*, right eye; *L*, left eye; *a* and *b*, portions of left eye successively covered. B. Tracings of paths in front of window; *1* and *2*, normal; *3*, right eye covered; *4*, right eye and *a* of left eye covered; *5*, *6* and *7*, right eye and (*a* and *b*) of left eye covered; large arrows, mean direction of light from window; small arrows, direction of locomotion. Note that with the right eye and the lateral surface of the left eye covered the insect went fairly directly toward the light.

make continuous circus-movements if one of the front legs is removed. Numerous observations were made on a considerable number of specimens with but one front leg and it was found that orientation, even immediately after the operation, was practically as accurate as it is in normal specimens (fig. 4). If the light was moved to the right or the left these specimens turned directly toward it, to the right or the left. And it could be clearly seen that the action of the front leg was prominent in this turning. In the one case it was thrust out sidewise, attached and then flexed, pulling the

insect toward the normal side. In the other it was thrust forward and inward toward the median plane, attached and flexed, pulling the insect toward the mutilated side (fig. 5).

If the front and the middle legs on one side are removed orientation is still possible although there is much difficulty in locomotion. With these legs removed the insect usually goes fairly directly toward the light for some distance then deflects toward the normal side. Then, after having turned through an angle of about 60°, it attempts to turn toward the light

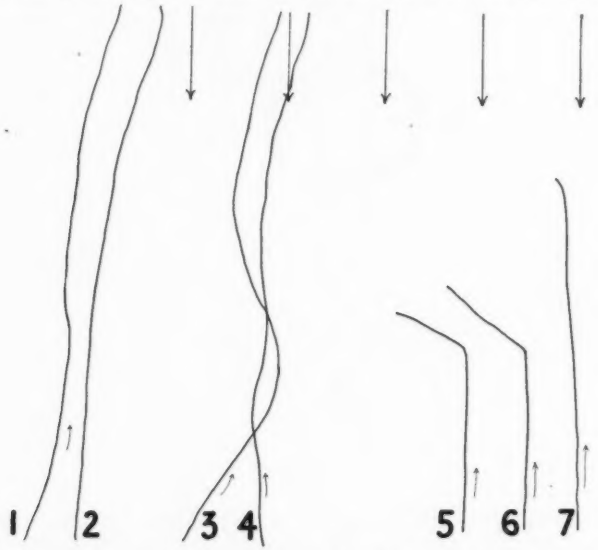

Fig. 4. Tracings of paths of Proctacanthus in front of window. *1* and *2*, individual normal; *3* and *4*, left front leg removed; *5*, *6* and *7*, right front and middle legs removed. Large arrows, mean direction of light from window; small arrows, direction of locomotion. Note that Proctacanthus with one front leg removed goes practically as directly toward the light as it does when normal, and that with two legs removed it goes fairly directly toward the light for a short distance, then deflects toward the normal side. After deflecting to the left the specimen represented attempted to turn sharply to the right and toppled over to the right at the end of each path.

but in this it usually fails, falling over toward the mutilated side (fig. 4). In going directly toward the light the front and the middle legs are continuously held much nearer the median plane than they are normally. During locomotion both are thrust well forward. In this movement the front foot reaches a point almost directly in front of the middle of the head where it is attached to the substratum and when the leg is flexed the insect is pulled almost straight forward. Immediately after the operation the

hind leg on the mutilated side appears to be partially paralyzed. It is merely dragged along, there being no stepping response in it, and it often relaxes to such an extent that the insect topples over. As time goes on this condition improves, so that the insect walks much better a day or more after the operation than it does immediately after.

These responses show very clearly that orientation occurs under conditions which exclude balanced or antagonistic action in the locomotor appendages on opposite sides and they indicate that impulses which originate in either eye pass to and control the action of the legs on both sides.

Discussion. In the preceding pages and elsewhere it has been demonstrated that the Ray-Verworn theory of orientation, the tonus hypothesis

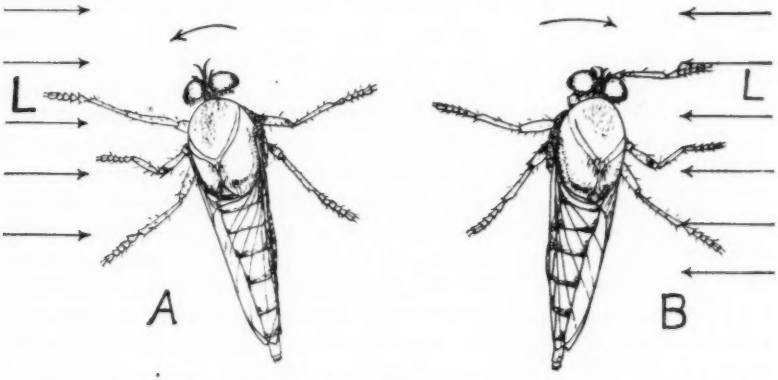

Fig. 5. Proctacanthus with one front leg removed. A, turning to the left toward the light. B, turning to the right toward the light; *L*, beam of light. Note the positions of the front leg. It is used in pulling the animal to the right as well as to the left.

or any other hypothesis that demands balanced or antagonistic action in the receptors and the locomotor appendages on opposite sides is inadequate to explain the process of orientation in insects for it is not in accord with the following phenomena:

1. Orientation with the two eyes continuously unequally illuminated. *a*, One eye covered. *b*, One eye and the posterior portion of the other covered. *c*, In light from two sources of the same size and at the same distance but different in intensity.

2. Orientation in specimens with some of the legs on one side removed.

3. Turning upward or downward while the two eyes are continuously equally illuminated. This occurs when insects on the wing turn upward or downward toward a source of light (Mast, 1923).

4. Turning in one direction and leaning in the opposite direction in insects with one eye covered or with the upper portion of one and the lower portion of the other covered.

5. Degree of deflection independent of intensity in one-eyed specimens in a horizontal beam of light (Dolley, 1916).

6. Decrease in deflection with increase in illumination in one-eyed specimens subjected to nondirective light from above (Dolley, 1916).

These phenomena are of fundamental importance in the process of orientation. It consequently seems evident that any theory worthy of consideration should be in accord with them.

In an earlier paper (Mast, 1923) it was concluded that orientation in insects is brought about by series of coördinated reflexes, the character of which is determined by the location and the integration of the stimuli in the eyes. It was maintained that stimulation of the ommatidia at the postero-lateral surface of one eye induces forward stepping in the legs on one side and backward stepping in those on the other side with certain sidewise deflections which differ radically in the different legs; that the sidewise deflection decreases as the stimulus is moved forward in the eye until it becomes zero and the insect goes directly toward the light; that if both eyes are simultaneously stimulated the effect in one is profoundly altered by the effect in the other, it being neutralized if the two stimuli are the same in magnitude and in location or if one is greater than the other, provided the greater is farther forward than the smaller; and that the farther back in the eye a stimulus is, the greater the efficiency will be as measured by the effect on turning. It was also maintained that the various series of orienting reflexes specifically correlated with various localizations and integrations of stimuli are instinctive responses which probably originated in the same way that other instinctive responses originated.

These views are strongly supported by the fact that all of the responses referred to above are in accord with them, particularly the response consisting of simultaneously leaning in one direction and turning in the opposite direction. This response occurs most readily, as previously stated, in specimens in which the upper portion of one eye and the lower portion of the other are covered.

If specimens with the upper portion of the right eye, e.g., and the lower portion of the left eye covered, are placed on a white background so that the left side faces the light, they lean toward the right and turn toward the left. If now the right eye is entirely covered they turn to the left without leaning to the right but if the left eye is entirely covered they lean to the right without turning to the left. This seems to prove conclusively that when they simultaneously lean toward the right and turn toward the left, the leaning is the result of a response to stimulation of the lower portion of the right eye and the turning to stimulation of the dorso-lateral portion of the left eye. I can account for this reaction only on the assumption that we have here two distinct sets of coördinated reflexes proceeding at the same time, that all of the legs are involved in both, and that both are

directly correlated with the locations of the stimuli in the two eyes. Such responses are possible only when the location of the stimulus in the two eyes differs radically. Ordinarily, the effect of the stimulus in one merely modifies or neutralizes the effect of the stimulus in the other, as for example, when the stimuli in the two eyes are the same in location but differ in magnitude, or when they are the same in magnitude but one is farther back than the other.

Cole (1923) maintains that the fact that one-eyed organisms in non-directive illumination turn continuously toward the functional eye shows that this response cannot be due to the localization of the stimulus in the eye "since all parts of the retina are equally stimulated."

I have demonstrated fairly conclusively in this and in preceding papers that if in one-eyed insects, the stimulus is located in the anterior region of the eye, there is no turning but that if it is located in the lateral regions of the eye the insects turn, and the farther back in the eye the sharper the turning, provided the stimulus remains continuously the same in magnitude. If this is true what response would be expected if all of the regions of the retina of the eye are simultaneously and equally stimulated? Under such conditions stimulation of the anterior region would tend to induce movement straight forward, but stimulation of all of the lateral regions would tend to induce turning. The expected response would consequently be an integration between these tendencies, that is, continuous turning, or circus-movements, which is precisely what Cole observed in Limulus and what has been repeatedly observed by various other investigations in many other organisms (Mast, 1911, p. 215). It would also be expected that the rate of turning would decrease if the posterior portion of the functional eye is covered and increase if the anterior portion is covered. The former has been definitely established in the preceding pages; the latter has not been specifically investigated but judging from results obtained on the character of the response to localized stimulation in normal specimens there is every indication that it is valid. Circus-movements are consequently in full harmony with the localization concept.

SUMMARY

1. Proctacanthus is photopositive. It orients directly and fairly accurately. It tends to come to rest at short intervals and to remain motionless for long periods of time.

2. Blind specimens and normal specimens in very weak light assume postures and walk essentially like normal specimens in strong light. There is no observable difference in muscle-tonus. This statement holds for short exposure. The effect of long exposures was not ascertained.

3. Specimens with the front leg or the front and the middle legs on one side removed orient fairly well. This indicates that orientation is not

necessarily dependent upon balanced or antagonistic actions in the locomotor appendages on opposite sides, and that impulses from either eye control the action of the legs on both sides.

4. If the light is so situated in normal specimens that it is directly opposite the ommatidia at the postero-lateral border of one eye, the feet on one side move forward and those on the opposite side move backward, the two front feet deflecting toward the light, the two hind ones from it. This shows that orientation is not necessarily due to difference in the rate of movement of the feet on opposite sides.

5. Specimens with one eye covered usually lean toward the functional eye. On a white background leaning is much more marked than on a black background. This is due largely to illumination of the ventral surface of the eye, and it appears to be the result of an attempt to turn toward the surface of the eye illuminated. If the ventral surfaces of the two eyes are equally illuminated there is no leaning. The illumination of one inhibits the leaning effect of the illumination of the other.

6. Specimens with one eye covered tend to turn continuously toward the functional eye, i.e., in the direction toward which they lean, but if the lower half of one eye and the upper half of the other are covered, they may turn toward the former while they are leaning toward the latter. Leaning and turning are consequently not necessarily causally related.

7. If one eye and the ventral or posterior portion of the other are covered, leaving only the anterior region of one eye functional, the insects orient fairly accurately; and when they are oriented the two eyes are not equally illuminated. When normal specimens are oriented in light from two or more sources which differ in intensity but are equal in other respects the two eyes are also not equally illuminated. Since this condition is practically universal in nature it follows that when insects are oriented under natural conditions the two eyes are rarely if ever equally illuminated. Orientation in insects is consequently not necessarily dependent upon a specific relation in the amount of light received by the two eyes.

8. Orientation in Proctacanthus is the result of series of reflexes specifically related to the location of the stimulus in the eyes. Stimulation of different regions in either eye induces different series of reflexes in which all of the legs are involved. If both eyes are simultaneously stimulated, the effect produced in one is modified or neutralized by that produced in the other. If the stimuli in the two eyes are the same in location and magnitude, light has no immediate observable effect. If they are the same in location but different in magnitude, the insect turns toward the stronger. If they are the same in magnitude and different in location, the insect turns toward the one which is farther back. It is consequently evident that location of the stimuli in the eye is quite as important in the process of orientation as the relation in the amount of light received by them.

BIBLIOGRAPHY

BOHN, G. 1904 Compt. Reudus. Acad. Sci. Paris, cxxxix, 890.
1909a. Les Psychisme. Scientia., v, 1.
1909b. Les tropismes. Rapport Vime Congr. Internat. Psychol. Geneve.
CARPENTER, F. W. 1908. Journ. Comp. Neurol. and Psychol., xviii, 483.
COLE, W. H. 1923. Journ. Gen. Physiol., iv, 417.
COWLES, R. P. 1908. Publ. 103, Carnegie Inst. of Wash., 1.
DE CANDOLLE, A. P. 1832. Physiologie vegetale. Paris.
DOLLEY, W. L., JR. 1916. Journ. Exper. Zoöl., xx, 357.
GARREY, W. E. 1918. Journ. Gen. Physiol., i, 101.
HOLMES, S. J. 1905. Journ. Comp. Neurol. and Psychol., xv, 305.
JORDAN, H. J. 1922. Arch. Neelandaises de Physiologie de l'Homme et des Animaux, vii, 314.
LOEB, J. 1888. Sb. d. phys. med. Ges., Würzburg, 1.
1893. Arch. f. d. gesammt. Physiol., liv, 81.
1897. Ibid., lxvi, 439.
1905. Studies in general physiology. Chicago.
1906. The dynamics of living matter. New York.
1918. Forced movements, tropisms, and animal conduct. Philadelphia.
MAST, S. O. 1911. Light and the behavior of organisms. New York.
1922. Natl. Acad. Sci., viii, 240.
1923. Journ. Exper. Zoöl., xxxviii, 109.
MINNICH, D. L. 1919. Journ. Exper. Zoöl., xxix, 343.
MÜLLER, H. 1876. Flora, lix, 65, 88.
PARKER, G. H. 1903. The phototropism of the mourning-cloak butterfly, Vanessa antiopa Linn. Mark Anniversary Volume, 453.
RADL, E. 1903. Untersuchungen über den Phototropismus der Tiere, Leipzig.
VERWORN, M. 1894. Allgemeine Physiologie. Jena.

CIRCULATORY RESPONSES TO DISTURBANCES OF THE STOMACH

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Clinicians have long known that diseases of the gastro-intestinal tract may give rise to circulatory and respiratory symptoms. Such symptoms may arise as the result of mechanical irritation or of absorption of digestion products. The present paper deals with the condition of the circulation and respiration following disturbances in the stomach. Dogs, under morphine-urethane anesthesia, were used in all cases. The experiments were directed to a study of the effects of inflation and mechanical, electrical and chemical irritation of the walls of the stomach.

Inflation. A thin-walled rubber balloon, inserted in the stomach cavity, was used to accomplish inflation. The response to this procedure was the same with the stomach *in situ* and with it lifted from the abdominal cavity and wrapped in warm, moist cloths. Simultaneous records of the arterial blood pressure, respiration and intragastric pressure were obtained. The records showed an increase in pulse rate, a rise in blood pressure and an increase in amplitude and frequency of respiration. The following experiment is given as an example.

Exper. 15. Male terrier, weight 9.2 kilos. Morphia-urethane anesthesia. Duodenum cut between ligatures. Stomach lies without traction. The data from this experiment are given in table 1.

Other similar experiments indicated that an intragastric pressure of from 10 to 20 mm. Hg was necessary to elicit such responses. All parts of the stomach are apparently responsive to inflation pressure because when the cardia was separated from the pylorus by ligature and the balloon inflated in first one and then the other cavity the results were essentially the same.

Similarly the outcome is the same whether the intragastric pressure is raised intermittently or continuously. The quickness and extent of the pressure were, however, significant factors. A sudden stretch of the tissues was essential to obtain the response. If the tone of the stomach relaxed the response disappeared. The importance of the quick rise of pressure was emphasized in experiments in which the balloon

was slowly filled with water. No circulatory or respiratory response followed although the stomach so distended was still irritable as indicated by the fact that electrical and chemical irritation of the serosa called forth a marked response (see below).

Chemical irritation. The application of mustard or of hydrochloric acid (5 per cent) to the mucous membrane was without effect. When applied to the serosa however unqualified positive results were obtained. Barium chloride (5 per cent) and sodium carbonate (saturated) likewise gave positive results when applied to the serosa.

TABLE 1

TIME IN SECONDS	PULSE-RATE PER MINUTE	RESPIRATIONS PER MINUTE	AMPLITUDE RESPIRATIONS	ARTERIAL PRESSURE IN MM. Hg
1–10	36	0	0	152
11–16	50	20	19	170
17–26	36	30	2	164
27–36	36	30	1	170
Inflation commenced				
37–42	80	110	30	200
43–48	110	120	32	230
Inflation completed				
49–58	120	132	70	240
59–67	120	122	70	240
Air let out				
68–73	90	100	65	240
74–81	90	105	52	220
82–91	84	108	50	230
92–101	78	102	35	210
102–111	78	108	25	220
112–121	60	106	12	200
122–131	42	100	3	186
132–141	42	72	3	186
142–147	40	60	1	186

Mechanical and electrical irritation. Clean-cut responses were obtained in every case. In the experiment, the data of which are given in table 2, the stomach was suspended by threads so that it hung free of contact with other structures. Otherwise the conditions were as before.

On the basis of what has gone before it can be stated that all forms of stimulation of the gastric serosa result in circulatory and respiratory changes which are sharp and decisive.

A stomach distended slowly with water, as has been mentioned, does not give rise to circulatory and respiratory changes. If, however, in

TABLE 2

TIME IN SECONDS	PULSE-RATE PER MINUTE	RESPIRATIONS PER MINUTE	AMPLITUDE RESPIRATIONS	ARTERIAL PRESSURE IN MM. Hg
1–6	80	0	0	235
7–12	70	0	0	230
Pinched serosa—1 second				
13–16	90	90	19	275
17–20	75	120	28	250
21–26	90	100	8	235
27–32	90	100	6	230
33–37	99	36	7	230
38–41	105	75	1	232
Rubbed serosa with pincers—1 second				
42–44	100	80	13	250
45–47	120	140	24	235
48–49	90	120	7	240
50–54	84	84	1	230
55–60	100	30	4	230
61–70	100	0	0	230
71–82	96	0	0	230
Electrical stimulation of cardia—electrodes 2 cm. apart				
83–86	90	15	1	240
87–90	120	120	18	250
91–92	100	140	22	275
93–95	150	120	19	250
96–97	120	90	12	275
Stimulation stopped				
98–99	120	120	8	245
100–103	120	120	14	230
A period of rest				
213–218	70	0	0	230
219–224	70	0	0	230
Rubbed serosa—6 seconds				
225–228	60	0	0	230
229–230	90	90	13	240
231–233	160	140	23	270
234–236	160	160	23	260
237–240	150	135	25	250
241–246	150	105	14	230
247–250	120	20	1	225

such a preparation peristaltic waves arise either spontaneously or as the result of stimulation each of such waves is accompanied by changes in the circulation and respiration. In the course of four experiments thirty pronounced peristaltic waves were observed. Twenty-five were accompanied by an increased pulse-rate; seventeen by a rise in blood pressure; twenty-one by an acceleration of respiration and ten by an increased respiratory amplitude.

In this connection it is of interest to note Carlson's observations on normal men and animals (1). He noted an acceleration of the pulse and vasomotor changes which occurred synchronously with gastric contrac-

TABLE 3

TIME IN SECONDS	PULSE-RATE PER MINUTE	RESPIRATIONS PER MINUTE	AMPLITUDE RESPIRATIONS	ARTERIAL PRESSURE IN MM. Hg	INTRAGASTRIC PRESSURE MM. Hg	PERISTALTIC WAVES
1–2	60	40	1	196	14	−
3–5	120	40	1	210	16	+
6–7	90	30	1	196	13	−
8–9	90	30	8	196	12	−
10–11	60	30	1	202	13	−
12–14	100	40	4	210	18	+
15–24	66	30	2	200	12	−
25–27	80	40	1	196	13	−
28 35	75	30	1	202	13	−
36–38	120	40	1	210	16	+
39–48	60	24	2	200	15	−
49–58	66	24	2	200	15	−
59–67	60	20	3	200	15	−
68–69	90	30	1	210	19	+
70–72	120	40	1	210	18	−
73–75	100	20	10	200	18	−
76–85	66	30	1	200	18	−
86–87	90	60	1	200	18	−
88–90	100	40	2	210	20	+
91–100	60	30	2	205	19	−
101–107	60	26	2	205	19	−

tions. Doubtless, as in the present experiments, the gastric musculature is responsible for the changes which are mediated through the sensory nerves in the serosa. Carlson and Luckhardt (2) have also reported reflex cardiac inhibition and acceleration from stimulation of visceral afferent nerves (including the alimentary tract) in amphibia and reptilia.

The following experiment is confirmatory of Carlson's observations. Into the stomach, lifted out of the body cavity, 500 cc. of water were introduced. Barium chloride (5 per cent) was then cautiously applied to the serosa. The peristaltic waves which resulted were noted on the records as indicated in table 3.

Pathway of afferent fibers. In four experiments the esophagus was cut below the diaphragm. Inflation of the stomach still gave the usual responses. Hence the vagus fibers are not essential for the reflex. It would thus appear that the afferent fibers lie in the sympathetic system. But there is a possibility that the vagi also contain afferent fibers. Stimulation of the vagus fibers isolated from the stomach wall gave, in the main, negative results (eighteen experiments on eight dogs). On one dog, however, on two occasions stimulation of these fibers resulted in a decreased pulse-rate (36 and 10 beats) a rise in arterial pressure (44 mm. Hg) and an increase in respiratory rate and amplitude.

In another experiment the stomach was cut free of all connections with the body except for the esophagus, thus leaving only vagus sensory fibers; three dogs were thus prepared. In two of these, inflation of the stomach slowed the pulse, raised the arterial pressure and increased the amplitude and frequency of respirations but to an extent very much less than when the sympathetics are intact. It would seem, therefore, that the easiest path for the reflex is over the sympathetics although it is possible for the impulses to travel over the vagus fibers as well.

Acceleration of the heart is the rule following gastric inflation but retardation occasionally occurs. Thus in four of eighteen inflations on nine dogs a slight but distinct slowing of the pulse occurred.

In the operative and experimental procedures of these experiments the animals not infrequently responded as if to painful stimuli. Since such responses followed procedures which elicited the reflexes under consideration it might be inferred that the seat or origin of the painful sensations is the gastric muscle.

While the writer, of course, accepts the view that disturbances in the hollow viscera may elicit pain the possibility must be borne in mind that the responses observed in the present experiments which have been interpreted as pain sensation may in fact be simple skeletal reflexes. If they are true pain sensations then it follows that the sympathetic nerve fibers must convey afferent impulses because the reflexes which we have discussed are only obtained occasionally when the sympathetic nerves are cut.

SUMMARY

Inflation of the stomach and mechanical, chemical and electrical irritation of the gastric serosa are followed by definite cardio-vascular and respiratory symptoms; the heart-rate is accelerated, the arterial blood pressure is raised and the respiration is increased in both rate and amplitude. These reflexes arise in the gastric musculature and are mediated chiefly but not wholly by the sympathetic fibers.

BIBLIOGRAPHY

(1) Carlson: This Journal, 1913, xxxi, 318.
(2) Carlson and Luckhardt: This Journal, 1921, lv, 31, 366.

THE EFFECT OF LUMINOUS INTENSITY ON THE RELATION BETWEEN STIMULATING EFFICIENCY AND FLASH-FREQUENCY OF INTERMITTENT LIGHT IN THE DRONE FLY, ERISTALIS TENAX.

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Contribution from the Marine Biological Laboratory, Woods Hole, the Biological Laboratory, Randolph-Macon College, and the Zoölogical Laboratory of The Johns Hopkins University

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It has been demonstrated in observations on the butterfly, Vanessa antiopa, and the tachina fly, Archytas aterrima, that the stimulating efficiency of intermittent light depends upon the flash-frequency and that it may be greater than, less than, or equal to that of continuous light (1), (2).

Mast (3) maintains that the fact that intermittent light has at certain flash-frequencies a higher stimulating efficiency than continuous light indicates that the processes involved in stimulation are not continuous, that there are in the nervous system alternate sensitive and refractory periods, stimulation occurring during the former and restitution during the latter. He holds, moreover, that a given quantity of energy must be received by the receptors during each of the sensitive periods and that the length of these periods consequently depends upon the luminous intensity,—the higher the intensity the shorter the period. If all this be true, then it is evident that the flash-frequency for maximum stimulation ought to be higher in strong light than in weak light. The experiments described in the following pages deal primarily with this problem.

Eristalis tenax was selected for the work because it can readily be procured and kept in excellent condition, and because it responds promptly and definitely and orients very precisely.

METHODS. At Woods Hole Eristalis was found in abundance on various flowers during the summer months. Here it was caught by net and taken to the laboratory where after clipping the wings it was kept in darkness to avoid excessive activity. A little dry granulated sugar occasionally was thrown into the finger bowls containing the flies. This was eaten freely. No water was added, but this would probably be required in drier regions. The flies remained in very good condition for a number of days so that the same individuals could be used day after day in extended series of observations.

The observations were all made on a table in a dark room in a field of light composed of two horizontal beams produced by two 100, 400 or 1000 watt stereopticon lamps so situated that the beams crossed at right angles. One of the lamps was mounted on a track in a long light-tight box so that the illumination could be easily varied by moving it back and forth. The other lamp was mounted in a light-tight cubical box which was placed on a small table. The boxes were painted dead black inside so as to reduce reflection, and each contained an opening which was screened in such a way as to produce a well-defined beam of light. Other screens were set up at various intervals in the two beams of light so as to make the field of light where they crossed 14 cm. square. The scattering of light by reflection was everywhere reduced as much as possible by means of dead black paper and velvet curtains. A rotating sectored disk was interposed in each beam. One of these was run at high speed (approximately 125 revolutions per second) by a motor connected directly with the lighting system. The other was run by a motor connected with an Edison storage battery with an adjustable rheostat in the circuit so that the rate of rotation could readily be varied and accurately controlled (fig. 1). The two disks were precisely the same, one-fourth being removed in both. The dark periods were consequently in all cases three times as long as the light periods. At the intersection of the two beams there was thus produced a field of light consisting of intermittent rays entering at right angles, the flash-frequency of those from one direction being constant and very high, and that of those from the other direction varying as desired.

It is known that if the light in the two beams in such a field is constant and equal, Eristalis will go toward a point half-way between them, and that if it is unequal it will go toward a point nearer the more intense beam. It may consequently be concluded that whenever the flies go toward a point half-way between two horizontal beams of light crossing at right angles the stimulating effect of the light in these beams is equal, regardless of the relation in quality or quantity. On the basis of this conclusion it was definitely proved in a preliminary set of tests that, with the illuminations used in the following experiments, the stimulating efficiency of intermittent light with a flash-frequency of 125 or over per second is practically equal to that of continuous light. Consequently, since the flash-frequency of the light in one of the beams in the field was always higher than this, it is evident that the effect of the light in this beam was the same as it would have been if it had been continuous and in the following experiment we shall, for the sake of simplicity, refer to it as continuous.

Intermittent light of high flash-frequency was used in place of continuous light in this beam because this made it possible to have the two

lamps at the same distance from the field of observation resulting in images in the two eyes of the same size; and it also greatly simplified measurements and adjustments necessary to insure equal illumination from them. To obtain this it was only necessary to ascertain the candle power of the two lamps, adjust them so that the luminous intensity produced by them at the center of the field was equal and insert the rotating sectors. Under these conditions the amount of light received from the two beams per unit of time remained equal regardless of the relation in flash-frequency in them. Consequently, to ascertain the effect of flash-frequency on stimulating efficiency it was merely necessary to change the rate of rotation of one of the sectored disks; and to ascertain the effect of intensity it was only necessary to change the distance of the two lamps from the field and to note the direction of movement of the flies in the field. For, as previously stated, if their path bisects the angle between the beams the stimulating effect of the light in them is equal and if it does not it is unequal.

The experiments were carried out as follows: The two lamps and the rheostat were adjusted so as to produce the intensity and the flash-frequency desired. A sheet of jet black paper (25 × 25 cm.) was placed at the intersection of the two beams on a horizontal platform slightly lower than the lower edge of the luminous filaments in the lamps; after which the field of light, where the beams crossed, was outlined with a pencil indicating the direction of the rays in each, and a line drawn bisecting the angle between these beams. A fly which oriented accurately in a single beam of light was now selected and placed facing the light in one of the beams near the inner edge and about 5 cm. from the corner of the field farthest from the sources of light (fig. 1 *x*). Its path was recorded by following it with a black pencil which was long enough to make it possible to keep the hand above the beams so as not to affect the direction of movement by reflected light. After it had crossed the field it was allowed to walk on a small piece of black cardboard with which it was transferred to the other beam and another path made and recorded as before. Thus the specimen selected was allowed to make 4 paths, beginning each one in the same relative place alternately in the two beams so as to neutralize the effect of entering the field from one side. Upon the completion of these 4 trials the fly was placed in darkness, the black sheet replaced by another, the flash-frequency changed to 200 per second and then, after the specimen had been in darkness 10 minutes, 4 more trials were given as before to serve as a control. This was repeated until series of paths were obtained for flash-frequencies of 66, 50, 40, 33, 25, 20, 14, 10, 5 and 2 per second with a series of control paths for each. The whole process was repeated with this specimen in other intensities and with other specimens in various intensities as indicated in tables 1 and 2.

RESULTS AND CONCLUSIONS. The results obtained are presented in tables 1 and 2 and in figure 2. In tabulating these results the angle of deflection from the line bisecting the angle between the beams was ascertained for each path. All those in which the deflection was toward the continuous light were labeled *c* and all those in which it was toward the intermittent light, *i*. Consequently in all paths with angles of deflection labeled *c* the stimulating effect of the continuous was greater than that of the intermittent light, and in all labeled *i* it was less.

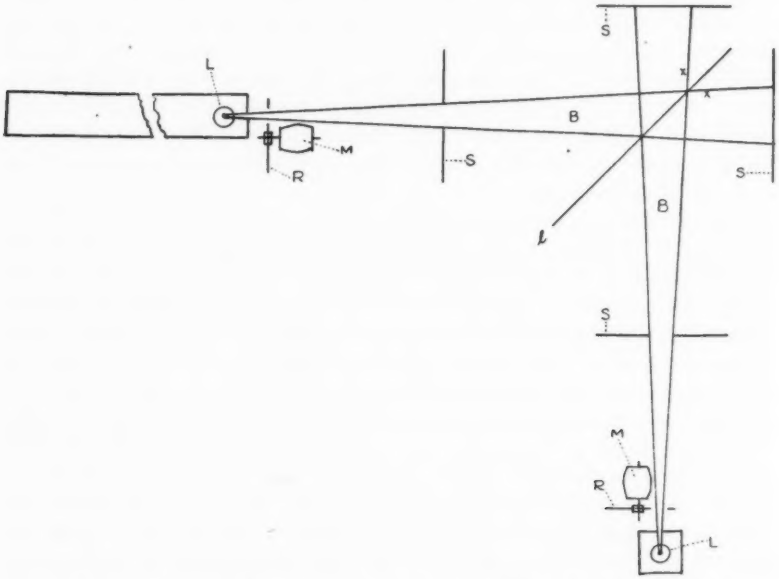

Fig. 1. Arrangement of apparatus used in ascertaining the effect of luminous intensity on flash-frequency for maximum stimulating efficiency. *L*, lamps in light-tight boxes; *R*, rotating sectors; *M*, motors; *S*, screens; *B*, beams of light; *l*, line bisecting angle between beams of light; *x*, *x'*, starting points in all trials.

By referring to these tables and the figure it will be seen at once that in any given illumination the angle of deflection depends upon the flash-frequency. Take, for example, specimen I in 227 m.c. Here we find that as the flash-frequency decreased from 66 to 2 per second the angle of deflection increased from 4.87° toward the intermittent light at flash-frequency of 66 to 17.62° at *25* and then decreased to 7.5° at *10*, after which it increased to 18° toward the continuous light at *2*. At a flash-frequency of 200 per second the angle remained nearly zero throughout the whole series of tests, the average for the different sets of tests at this

flash-frequency, beginning above, being 2.12° *i*, 8.75° *i*, 3.37° *c*, 4.12° *c*, 7.62° *c*, 3.75° *c*, 6.50° *c*, 1.20° *c*, 0°, 4.87° *c*; total average, 2.05° *c*.

A further study of the tables shows that the results obtained in all of the tests are in harmony with this. These results support the conclusion reached by Dolley (1), (2) in experiments on Vanessa and Archytas, namely: that the stimulating effect of a given amount of intermittent light is at certain flash-frequencies greater than that of the same amount of continuous light.

TABLES 1 AND 2

Relation between flash-frequency, stimulating efficiency and luminous intensity. The numbers indicate the degree of deflection from the line bisecting the angle between two beams, one of continuous and the other of intermittent light. *i*, deflection toward the source of intermittent light; *c*, deflection toward the source of continuous light. The numbers under "average angle of deflection" are averages of 4 trials in all cases except those following flash-frequency 200 which are averages of 32 or more trials. Illumination in the two beams equal in all tests.

Compare the total average angles of deflection in the different intensities and note that the stimulating efficiency of intermittent light depends upon the flash-frequency and upon the intensity.

TABLE 1

FLASH FREQUENCY PER SECOND	AVERAGE ANGLE OF DEFLECTION DESIGNATION OF FLIES					TOTAL AVERAGE ANGLES
	D	E	A	C	B	
Illumination 550 m.c.						
66	2.00 c	6.37 c	5.25 c	12.12 c	1.62 i	4.82 c
50	13.62 i	5.12 c	6.25 i	9.75 c	4.50 c	0.10 i
40	5.00 i	11.12 c	10.75 i	21.62 i	2.12 c	4.82 i
33	11.50 i	9.37 i	21.62 i	29.00 i	12.00 i	**16.69 i**
25	1.87 i	1.50 i	25.62 i	33.00 i	4.50 i	**13.29 i**
20	2.87 i	0.75 i	13.12 i	3.00 c	15.62 i	5.87 i
14	5.50 c	6.50 c	22.75 i	0.87 i	6.87 i	3.69 i
10	22.75 c	24.50 c	11.25 c	21.87 c	0.12 c	16.09 c
200	9.78 c	11.32 c	1.51 c	0.96 c	2.84 i	4.14 c
Illumination 9.46 m.c.						
66	5.37 c	5.37 c	1.00 c	2.12 c	5.25 i	1.72 c
50	0.87 i	15.87 c	19.87 i	3.62 c	7.12 i	1.67 i
40	3.25 c	8.25 c	2.50 c	7.12 c	7.62 c	5.74 c
33	10.62 c	18.37 c	8.75 i	5.00 c	6.37 i	3.77 c
25	8.50 c	8.25 c	1.62 c	0.62 c	9.12 i	1.97 c
20	2.00 i	2.62 c	4.50 i	2.00 i	15.25 i	4.22 i
14	20.87 i	9.37 c	5.00 i	20.75 i	3.50 i	**8.15 i**
10	3.62 i	9.87 c	9.00 c	17.37 i	14.50 i	3.32 i
200	7.65 c	13.57 c	3.09 i	6.20 c	0.39 i	4.78 c

TABLE 2

FLASH FREQUENCY PER SECOND	AVERAGE ANGLE OF DEFLECTION DESIGNATION OF FLIES					TOTAL AVERAGE ANGLES
	E	K	L	I	M	
Illumination 227 m.c.						
66	6.12 c			4.87 i	4.37 i	1.04 i
50	19.85 c	3.50 i	5.62 c	5.12 i	31.12 i	2.85 i
40	16.12 c	0.87 i	0.62 i	11.62 i	21.12 i	3.62 i
33	8.62 c	6.75 i	12.37 i	14.75 i	13.00 i	7.65 i
25	0.75 i	17.75 i	13.12 i	17.62 i	27.37 i	**15.32 i**
20	5.62 i	18.87 i	7.00 i	11.75 i	18.50 i	**12.34 i**
14	14.62 i	4.62 i	0.50 c	13.12 i	14.25 i	9.22 i
10	8.50 c	9.12 c	6.62 c	7.50 i	10.00 i	1.34 c
5	1.50 c	7.12 c	21.62 c	9.12 c	25.87 c	13.04 c
2	8.87 c	22.00 c	26.00 c	18.00 c	16.00 c	18.17 c
200	1.98 c	1.19 c	4.19 c	2.05 c	3.13 c	2.50 c
Illumination 92.19 m.c.						
50	0.37 i	0.75 i	3.37 i	5.37 i	4.12 i	2.79 i
40	2.12 i	4.12 c	16.00 c	16.62 i	5.75 i	0.87 i
33	5.62 i	7.75 i	2.00 i	14.37 i	9.00 i	7.74 i
25	6.37 i	5.75 i	3.87 c	12.62 i	19.00 i	7.97 i
20	7.25 i	16.37 i	5.37 i	20.37 i	7.75 i	**11.42 i**
14	5.12 i	10.12 i	6.75 i	23.00 i	8.00 i	**10.59 i**
10	6.50 c	6.50 i	11.62 c	2.37 i	8.62 i	0.12 c
5	11.25 c	26.37 c	29.87 c	12.12 c	23.12 c	20.54 c
2	16.75 c	29.37 c	24.16 c	1.12 i	18.87 c	17.60 c
200	1.26 i	5.52 c	5.97 c	1.40 i	7.90 c	3.34 c

If the averages for the angle of deflection in different luminous intensities are compared it will be seen that the flash-frequency for maximum deflection toward the intermittent light differs greatly, it being approximately 30° in 550 m.c., 23° in 227 m.c., 18° in 92.19 m.c. and 14° in 9.46 m.c. (fig. 2). This indicates that the higher the intensity is the higher the flash-frequency for maximum stimulating efficiency will be.

These results are in complete harmony with the hypothesis previously presented, that there are in the receptors or the nervous system of insects alternate sensitive and refractory periods, and that in continuous illumination light does not act continuously but only during the sensitive periods, restitution occurring during the refractory periods. In accord with this hypothesis the illumination of a photo-receptor results in certain changes, probably photochemical in nature; these proceed in a certain direction and continue until a quantitatively definite amount of change

has occurred; then a reverse change sets in (restitution) regardless as to whether illumination continues or not. In continuous illumination the light which is received during the refractory period is consequently neutral in its effect. Intermittent light of such a nature that the dark periods

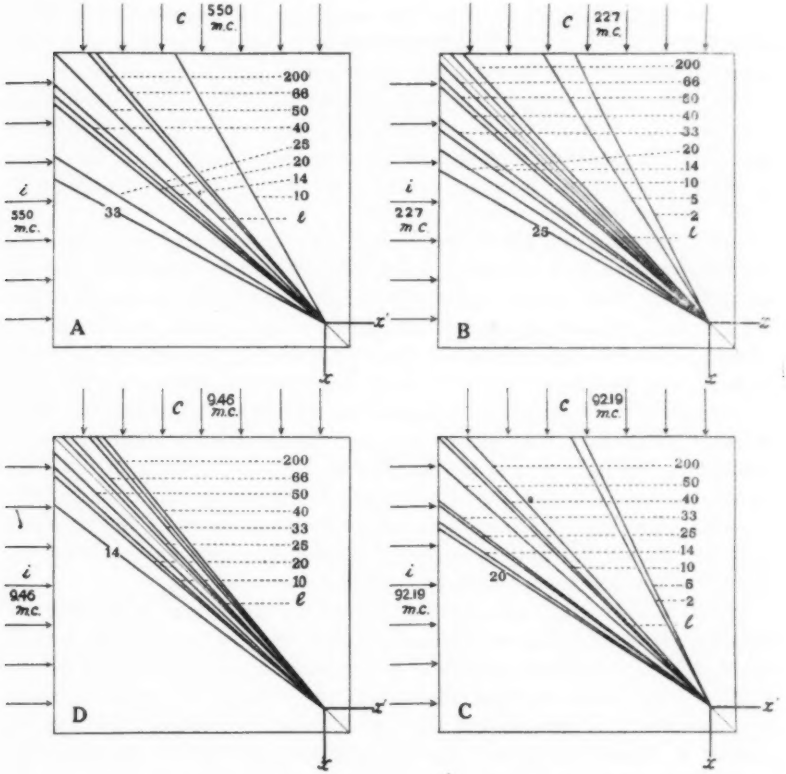

Fig. 2. Diagram showing average of paths made by Eristalis in a field of light composed of two horizontal beams of equal illumination crossing at right angles. *c*, continuous light; *i*, intermittent light; *l*, diagonal bisecting angle between beams; *2–200*, flash-frequencies of intermittent light and paths in each; *x*, *x'*, alternate starting points in all tests. Illumination from each beam at center of field: A, 550 m.c.; B, 227 m.c.; C, 92.19 m.c.; D, 9.46 m.c. Path 200 A, average of 160 trials; 200 B, 192; 200 C, 160; 200 D, 180; 66 B, 12; all others, 20.

Note that the deflection of the paths from the diagonal varies in each intensity with the flash-frequency of the intermittent light, and that for the same flash-frequency it varies with the intensity, the flash-frequency for greatest deflection toward the intermittent light, the optimum flash-frequency, being approximately 33 in 550 m.c.; 25 in 227 m.c.; 20 in 92.19 m.c. and 14 in 9.46 m.c.

coincide with the refractory periods therefore would be expected to have a higher stimulating efficiency than continuous light, i.e., the stimulating effect of a given amount of light received would be greater, and if the change induced by light during the sensitive period is specifically related to the amount of light received during this period it is evident that the more intense the light the shorter the sensitive period would be and consequently the higher the flash-frequency for maximum stimulating efficiency. This is precisely what was observed, as previously set forth.

A further comparison of the results presented in the tables shows also that the magnitude of the maximum deflection toward the intermittent light depends upon the intensity, it being approximately 16° in 550 m.c., 15° in 227 m.c., 11° in 92.19 m.c. and 8° in 9.46 m.c. That is, it is greatest in the highest intensity used.

If the length of the refractory period is independent of the luminous intensity, as would be expected, and the sensitive period increases as the intensity decreases it is evident that the difference between the stimulating efficiency of continuous and intermittent light would be less in low intensity than in high, provided there is a direct quantitative relation between the amount of light received during the sensitive period and the change induced by it, for the lower the intensity the shorter the dark period in relation to the light period; and the shorter the dark period in relation to the light period the smaller the relative amount of light received and wasted during the refractory period in continuous illumination. The fact then that the maximum stimulating efficiency of intermittent light in relation to that of continuous light decreases as the intensity decreases is in full accord with the hypothesis considered.

In further investigation now under way we shall deal quantitatively with the effect of luminous intensity on the relation between the length of the light and the dark periods in intermittent light of maximum stimulating efficiency and with the difference between the stimulating efficiency of continuous and intermittent light in different intensities. The results already at hand indicate that this investigation will further elucidate the processes involved in stimulation.

SUMMARY

1. The stimulating efficiency of intermittent light in the orientation of Eristalis varies with the flash-frequency, i.e., depending upon the flash-frequency the effect of intermittent light may be greater than, equal to, or less than that of continuous light of equal illumination.

2. In intermittent light the flash-frequency for maximum stimulating efficiency varies with the illumination. It is higher in strong light than in weak light. If this is true it follows that in insects exposed to continuous illumination, light does not act continuously.

3. The maximum stimulating efficiency of intermittent light in relation to that of continuous light decreases as the luminous intensity decreases.

4. These facts support the contention that there are in the photoreceptors or the nervous system of insects alternate sensitive and refractory periods and that continuous illumination does not act continuously in photic stimulation.

BIBLIOGRAPHY

(1) DOLLEY: Psychobiol., 1920, ii, 137.
(2) DOLLEY: This Journal, 1923, lxiv, 364.
(3) MAST: Journ. Exper. Zoöl., 1923, xxxviii, 109.

THE EFFECT OF PUBESCENCE, OESTRUATION AND MENOPAUSE ON THE VOLUNTARY ACTIVITY IN THE ALBINO RAT[1]

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In a former experiment (1) to determine the voluntary activity of the albino rat during its entire life span, marked rhythmic increases of activity followed by pronounced decrease were noted in the females. These spurts of activity occurred about every fourth day. Since no correlation could be made between these periods of activity and the food, the barometric pressure, the temperature or the humidity, we were led to conclude that some rhythmic change in the animal itself was the cause.

In order to throw more light on this subject and to determine the cause of this rhythmic activity, an experiment was started with eight female rats in April, 1918. Owing to the disorganization of our forces, the withdrawal of one of the experimenters, and increased duties caused by the late war, this experiment was much interfered with and unsatisfactory. A second experiment was therefore begun October, 1921, and has been continued to the present.

Eight young female rats which had not reached pubescence were also used in this experiment. Five of these were sisters and 59 days old; the remaining three were sisters and 37 days old at the beginning of the experiment. Each rat was placed in a separate revolving cage (previously described) (2), (3) which was its home during the experiment. The advantage of this cage is that the nest box, which hangs on the stationary axle, is just sufficiently large to allow the rat to sleep comfortably. Practically all the activity of the animal, therefore, is manifested in turning the revolving cage.

The rats were fed table scraps, cracked corn and water daily at the same hour. The automatic recording devices, which registered the exact number of revolutions each cage had been turned during the past 24 hours, were read at the feeding time. In this manner we were able to compute for each day the voluntary running activity of each rat, as

[1] This research has been conducted with the aid of the Department of Physiology and the Research Fund of Stanford University, and The Committee for Research on Sex Problems of the National Research Council.

represented by revolutions of its cage, and to note any rhythmic changes. The curves of daily activity were then plotted as shown in the accompanying figures. The relative humidity of the air of the room was recorded daily. The self-registering thermometer and barometer recorded the temperature and barometric pressure at all hours of the day. The weather conditions and the amount of precipitation were also recorded and files of the daily weather map showing conditions in surrounding regions were kept on file for consultation. In this manner we were able to determine whether changes in these environmental conditions had any influence on the voluntary activity of our animals.

The room in which the experiment was conducted was an unheated and well-ventilated attic room with a north slope to the roof. It had no windows to the outside and was therefore in subdued light during the day time. External light conditions could therefore have no influence on activity. The temperature was fairly constant, the greatest daily range between maximum and minimum temperatures being 12°C. and the smallest range 3°C. The average daily range was about 7°C. The highest temperature recorded was 31°C. during one of our warmest days, and the lowest 10°C. during the winter.

A careful study of the changes in environment in regard to humidity, temperature and barometric pressure showed little or no correlation with the rhythmic increase in the voluntary activity of the female albino rat. Since other environmental conditions remained practically constant we were led to conclude that these rhythmic fluctuations were due to normal changes occurring in the animal.

This rhythm was not manifested in sexually immature females, nor did it occur in females that had reached senility and passed the menopause. Neither was this rhythmicity seen in males unless the cages in which they were confined were closely adjacent to that of an active female. In this case the males showed a rhythm similar to that of the adjacent female, but the fluctuations in activity were not nearly so great as those of the female. These facts led us to conclude that the spurts in activity of the female were associated with the oestrual cycle.

Numerous investigators have shown that in the normal adult female rat oestruation occurs every four or five days during sexual life. The work of Long and Evans (4) shows that oestrum occurs in sexually mature female rats on an average of every four and one-quarter days. In 1376 observations cited they found the shortest period to be two days and the longest thirty-nine days. They attribute the lengthening of the oestrual cycle to handling, impaired respiration due to piling up in the cages while sleeping, under-nutrition, etc. They have also shown that oestruation may be delayed by mechanical stimulation of the cervix. By vaginal smears they have demonstrated the presence of five distinct cell condi-

tions and on this basis have divided the oestrual cycle into five stages. During the second stage, lasting about twelve hours, the animal is in heat and will accept the male. Ovulation occurs a few hours later in the third stage. We have correlated these results with our activity curves and find that on the peak of the curve the vaginal smear is composed usually of cornified cells and represents stage two. The animals used in their investigation were a cross between the albino rat and the gray rat. They were also relatively young rats, ranging between the average ages of 100 and 169 days. This may account for some differences in results as given by us. Miller (5) has found that the average oestrual cycle of the gray rat is five days. We will also show later that the length of the oestrual cycle varies with the age of the rat.

Wang (6) has recently verified the results of Long and Evans in his work on "the relation between 'spontaneous' activity and the oestrous cycle in the albino rat." He used fourteen young females and found that rhythmicity in activity was absent until they had reached the age of pubescence. After this age regular increases in activity, occurring approximately every fourth day, were shown by the females, but were absent in the males and in females after ovariectomy, mechanical stimulation of the cervix uteri, and during gestation. He concludes that the existence of the activity cycle depends on the periodic functioning of the ovaries. He also, by means of vaginal smears, correlates his work with that of Long and Evans and finds that the activity cycle occurs at oestrum, or the cornified cell stage. Our results substantiate in general those of Wang. We find, however, a marked increase in activity, as exhibited by revolutions, in our rats over his even though his cage was 5 inches less in diameter than ours. This we attribute to the fact that the compartment in which his rats slept was large enough to permit considerable movement that would not be registered.

Since he has dealt only with young animals he has left incomplete the effect of age on the length of the cycles and the menopause. This we have studied having kept the same animals under continuous observation for over 800 days.

It has long been known that the sex glands play an important part in the development of certain sexual characteristics in an animal. Especially is this true in regard to the sex organs and their proper functioning during sexual life. It is not known whether other glands of internal secretion have any influence on the sex glands. The work of Goetsch (7), which showed that extract from the anterior lobe of the pituitary body stimulated growth of the sex organs, has been disproved by the more recent investigations of Frank (8) and Sisson and Broyles (9). Later investigations may demonstrate that other glands of internal secretion may have an important influence on the sex glands either in their development or in their proper functioning.

The absence of oestruation in spayed mature females and its presence in normal mature females indicates that the ovaries are essential for its occurrence. The fact that oestrum does not occur in sexually immature females or in those which have passed the menopause indicates further that something in the functioning ovary is the cause. The further fact, as Marshall (10) and others (11), (12), (13) have shown, that oestruation will occur normally if the ovaries are successfully transplanted to another portion of the body of the animal, points to a substance in the nature of a hormone which is secreted by the ovary as the activating agent. Various parts of the ovary have been suggested as possible sources of this hormone. Fraenkel (14) advanced the idea that the corpus luteum might be the source. This has more recently been shown to be associated with cerfain uterine changes preparatory to implantation of the fertilized ovum. Marshall and Runciman (15), studying oestrus in the dog, conclude that in the recurrence of "heat" the corpora lutea and the Graafian follicles play no part, but that the interstitial cells are possibly concerned in the process. That this hormone is contained in the follicular fluid has been advanced by Frank (16). By injecting follicular fluid into two normal virgin rabbits he was able to induce uterine hyperemia. Since the ovaries of these rabbits had not been removed they may have had an influence in bringing about this result.

Conclusive proof that the hormone inducing oestruation is in the follicular fluid has recently been given by Allen and Doisy (17). By means of a hyperdermic needle and a suction bottle they aspirated relatively large quantities of follicular fluid from the ovaries of swine which were in heat. This they injected subcutaneously into spayed animals. Within forty-eight hours all animals so treated were in full oestrum as shown by vaginal smears. They later eliminated all follicular cells and ova from the fluid and got similar results. This shows that the hormone is in the fluid and is extra-cellular. It is our plan not only to repeat these experiments, but also to see if typical oestrus can be induced in old animals that have passed the menopause.

It has been previously shown (18) that oestruation may be greatly interfered with or completely suppressed by an inadequate diet. Evans and Bishop (19) have recently shown that oestruation may occur in animals deprived of certain foods capable of producing normal growth, but that they fail to reproduce young. Fertilization either fails to take place or the young are resorbed. They have designated this substance which is essential for normal reproduction vitamin X. This is found in lettuce, alfalfa, germ of wheat and many other foods. We thus see that a food which produces normal growth is insufficient for the propagation of the race.

Many other investigations which bear more or less directly on this subject might be cited. They agree, however, in general with the results already given.

In our experiments, sexually mature female rats show during their sexual life a marked rhythmic increase in voluntary activity approximately every fourth day. Before pubescence and after the menopause this rhythmicity is absent and the amount of activity is relatively small as compared with that during sexual life.

The character and the amount of activity varies not only with different individuals but also with age. It is also affected by the kind of food given. Experiments are now in progress to show the effect of gestation, lactation, mating unaccompanied by complete gestation, artificial stimulation of the cervix uteri, and ovariotomy; also the effect of compulsory work during gestation on the length of the gestation period and on the character, size and number of young, and of excessive breeding on the individual, on the duration of sexual life and on the life span. These results will be published when completed.

By means of vaginal smears and mating tests we have correlated our results with those of other investigators and find that the peak in the activity curve corresponds and is identical with the cornified cell stage, or the second stage of Long and Evans. At this time the female readily accepts the male. Though coitus sometimes occurs at other phases of the activity curve it is only at the peak, or a few hours before or after it, that we have found fertilization to occur. Our experience seems to indicate that the making of vaginal smears, due to the necessary handling, etc., of the animal, tends to disturb the regularity of oestruation. We feel, therefore, that the normal sequence is more accurately shown by activity curves than by any other method advanced, as it leaves the animal undisturbed by unavoidably varying conditions.

Effect of age. When rats are transferred from stationary cages to revolving cages, a few days to two or three weeks are required for them to become adjusted to their new environment. Young animals usually made this adjustment more readily than old ones and begin to show their typical activity within a week. Prior to pubescence the daily activity is relatively uniform in amount, ranging from a few hundred to two or three thousand revolutions. Figure 1 shows the curve of activity of rat 6. This rat was placed in the revolving cage at the age of 37 days. Little change in its activity occurred until after the age of 79 days. From this date there was a marked rise to the age of 83, which indicates the first oestrum. After this, peaks are seen in the curve each fourth day, each succeeding peak being higher than the preceding until a maximum height is reached about the age of 120 days.

Figure 6 represents the activity of rat 8, a sister of no. 6, from the age of 47 to 92 days. This rat had been in its revolving cage since the age of 37 days. At first this curve showed no marked changes in daily activity, though there was a gradual increase in average daily running. On the 59th day, however, there was a decided increase and this very likely represents the first oestrum. No mating tests nor vaginal smears were made so we cannot verify the above supposition. The second high point occurred five days later and undoubtedly represents oestrum. The succeeding peaks constantly increased in height and occurred with marked regularity of four day intervals. The highest point in the curve

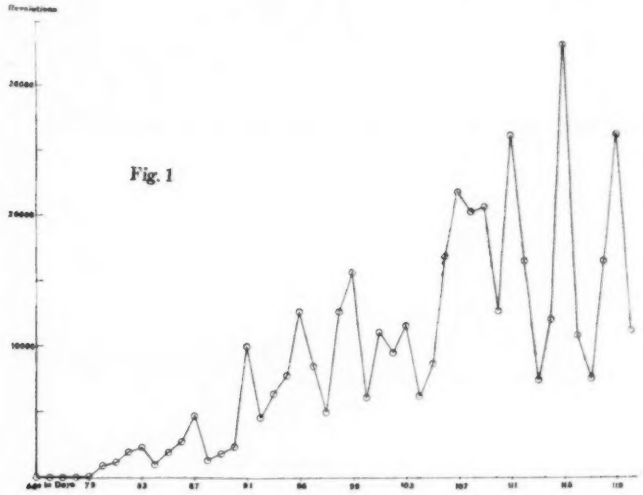
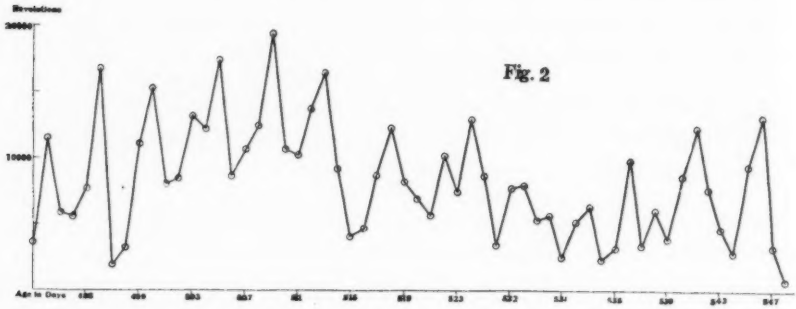


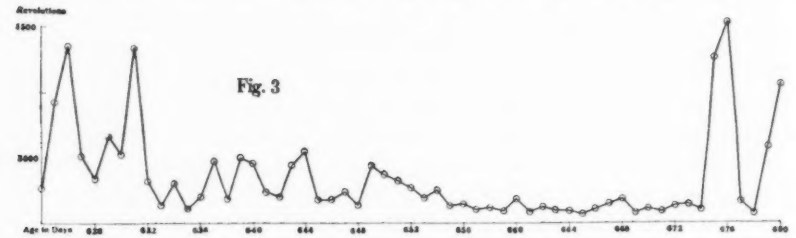
Fig. 1. Curve of activity of rat 6 from the age of 75 days to 120 days showing the beginning of its oestrual cycles. The high points indicate oestrum, the circles intervals of 24 hours.

of this rat was reached at the age of 122 days (fig. 7) when the cage was turned 42,980 revolutions in the preceding twenty-four hours. If the cage had rolled this number of revolutions in a straight line on the ground it would have covered a distance of 36.6 miles. Our record run for twenty-four hours is 44,640 revolutions or a distance of a little over 38 miles.

All our other rats showed very similar curves of activity during their early life and after pubescence to that described above. A variation, however, occurred in respect to the age of the first oestrum. This ranged from the age of 63 days to 109 days. (Tables 1 and 2.) Even in sister rats, subjected to the same environment, a difference of as much as 32 days was noted in the age at which the first oestrum occurred. All our

rats showed the gradual increase in the height of the peaks until they had reached approximately 120 days of age. At this age they all showed very marked differences between the peaks and the low points in their activity curves.





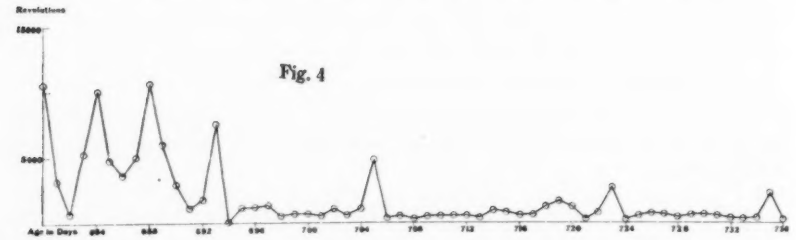


Fig. 2. Curve of activity of rat 6 from the age of 491 days to 548 days.

Fig. 3. Curve of activity of rat 6 from the age of 624 days to 680 days, showing approaching menopause.

Fig. 4. Curve of activity of rat 6 from the age of 680 days to 736 days, a short time before the menopause.

No regularity can be shown in regard to the distribution of activity between the peaks of oestruation and the low points. In some cases three days are required from the peak down to the low point and one day up to the peak, in others two days down and two up, while in still other

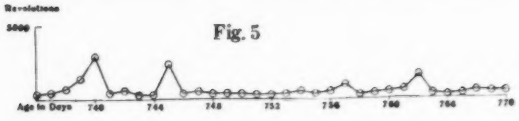


Fig. 5

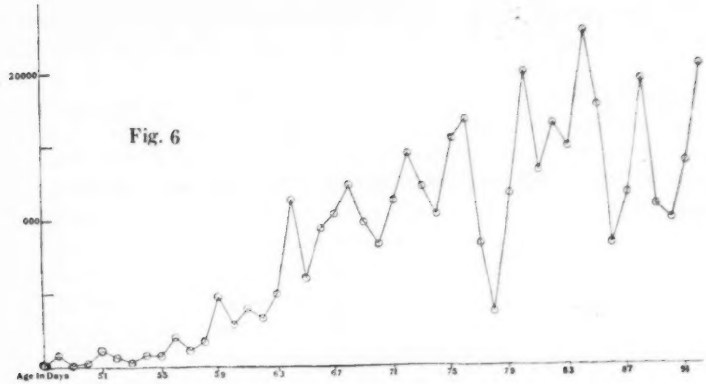


Fig. 6

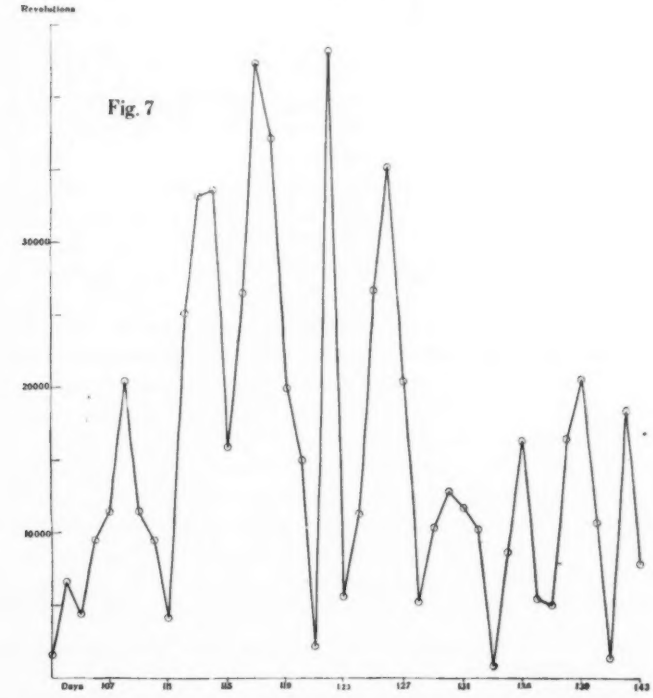
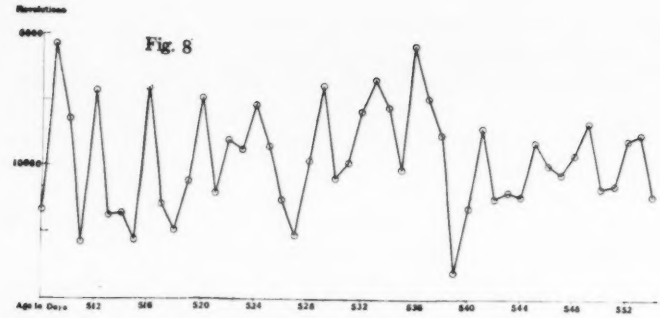


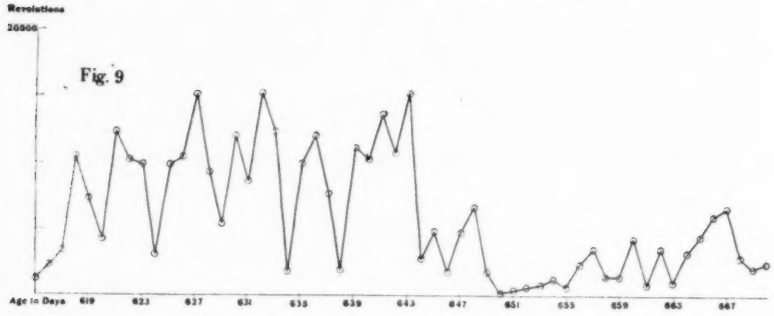
Fig. 7

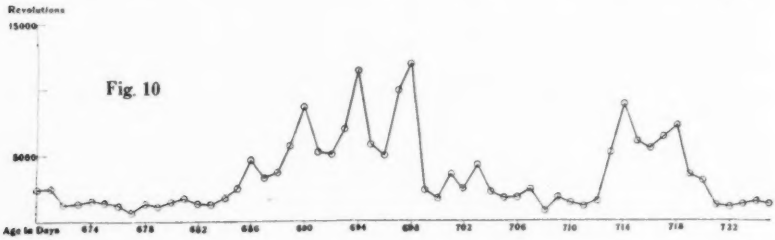
Fig. 5. Curve of activity of rat 6 from the age of 736 days to 770 days, showing the menopause.

Fig. 6. Curve of activity of rat 8 from the age of 47 days to 92 days, showing its first oestrual cycle at the age of 59 days.

Fig. 7. Curve of activity of rat 8 from the age of 103 days to 143 days, showing the period of greatest fluctuations.







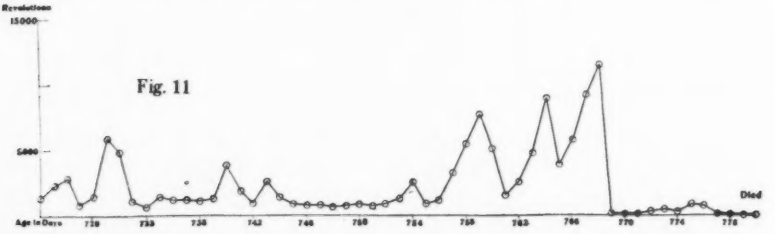


Fig. 8. Curve of activity of rat 8 from the age of 508 days to 554 days showing almost regular oestrual periods.

Fig. 9. Curve of activity of rat 8 from the age of 615 days to 670 days showing beginning of irregular cycles.

Fig. 10. Curve of activity of rat 3 from the age of 670 days to 725 days showing long dioestrual period followed by regular 4 day cycles.

Fig. 11. Curve of activity of rat 3 between the ages of 725 and 780 days, showing menopause at 769 days.

cycles we find one day down and three up. In other words, all possible combinations of activity occur in different cycles of the same rat. The low points, therefore, are not separated by equal intervals similar to the peaks. We have no explanation to offer for this irregularity in dioestrual ctivity.

As the animals grew older the heights of the peaks in the activity curve became gradually less and less and the differences between the high and low points less marked. This is shown in figures 2 and 8, which are the activity curves of rats 6 and 8 between the ages of 491 and 548 days, and 508 and 555 days respectively. These figures show a noticeable conformity to 4-day periods. Some irregularities, however, are seen as there were two 3-day periods and two 5-day periods in figure 8 and one 2-day, one 3-day and four 5-day periods in figure 2. By comparing these figures with figures 1 and 7, the effect of age is readily seen.

With advancing age there is a marked change in the character of the curve of activity. There is not only a general lowering of the amount of voluntary running, as we have previously shown (1), but there is a disturbance in the regularity of the oestrual cycle. This disturbance becomes more and more pronounced as the animals approach the menopause. Not only does the 4-day period become less common but oestrum may be entirely wanting for relatively long periods. As no two rats behaved in the same way, no definite statement can be made except that in general the oestrual cycles became more and more irregular and seem to have exerted a less influence on activity as the animals approached the end of their sexual life.

Figures 3, 4 and 5 represent the activity of rat 6 from the age of 624 days to 770 days. A typical oestrum occurred at the age of 631 days (fig. 3) and the next one at 676 days. If there were any between these ages the characteristic influence on activity was wanting. The curve shows that oestruation may possibly have taken place at the ages of 639, 644 and 649 days, but this is doubtful. After the age of 693 days an oestrum occurred only six, or possibly seven times in the 77 days intervening. The last oestruation occurred in this animal at the age of 762 days and exerted but a slight influence on the activity. Whether this is due to lack of the follicular fluid hormone, a reduction of its potency, to general senility, or individual characteristics of the animal, remains to be cleared up by our other investigations. Three other rats approached the menopause very similar to no. 6. Figure 11, which shows the last oestrum of rat 3, seems to indicate that the cause might be attributed to individual variation. The activity of three other rats during their last days of sexual life resembled rather closely that of no. 3. From these results one might reasonably infer that individual variation at least plays a part in influencing the character of the activity.

Figures 9, 10 and 11 represent the activity of rat 3 from the age of 614 days until its death at the age of 780 days. These curves show groups of three or four relatively regular periods, in which the effect of oestrum on activity is practically normal, separated by extended periods of varying lengths during which no characteristic oestrual effect is shown. In this animal it appears that oestruation practically ceased, but after a time reëstablished itself in almost normal sequence of four day periods for three or four cycles.

Rats 6 and 3 thus show two types of activity as effected by the termination of oestruation. Our other animals showed various intergradations between those two types, no two being the same.

EFFECT OF RESTRICTED DIET. When rats were fed an inadequate food, a marked effect on the activity and oestrual phenomena followed. This is illustrated by figures 12, 13 and 14, which are the activity curves of rat 3—1918, during the ages indicated. About 25 days previous to the beginning of curve 12 the food of the group of eight rats to which this one belonged was changed. Previous to this time the diet was selected from both animal and vegetable sources. At this date the food was selected from vegetable sources. About thirty days afterward the effect of the restricted diet began to be apparent (fig. 12). Oestrum continued to occur rather regularly and mainly at intervals of five days. The curve shows that there was a gradual reduction in oestrual effect on activity. After the age of 195 days the typical oestrual effect had practically ceased. Figure 13 shows the curve of activity of the same rat 42 days later between the ages of 255 and 311 days. This curve shows a few high points which were most likely due to oestrum. This figure resembles very closely the old age curve of rat 6 at the age of about 700 days, a short time before it had reached the menopause. The other rats of the group showed a similar effect. The diet thus caused senile conditions in relatively young rats, that on an adequate diet would have been about in their prime. Figure 14 shows the activity curve of this same rat about one month after the restricted diet had been replaced by an omnivorous one. Typical oestrual effects were again manifested and a rhythm, consisting mainly of 5-day periods, again established. This was true also in the other individuals of the group. In some of the rats the number of revolutions due to oestruation was greater after the resumption of an adequate diet than any exhibited in their earlier life. This bears out the statement that animals which have been retarded in various ways by an inadequate diet may be, within certain limits, practically restored to normal conditions if an adequate diet is administered.

LENGTH OF CYCLES. In order to show the length of the cycles at different ages and their sequence the data have been arranged in tabular form.

Table 1 gives the cycles in successive order of young rats from the first oestrus to the average age of 83.7 days. The youngest age at first oestrum, or pubescence, which occurred in these 22 animals was 46 days and the oldest age was 83 days. The average age of puberty was 64 days. As will be noted, the length of the first cycle ranged from 3 to 7 days and had an average of 4.5 days. The last observed cycle was more constant, ranging between 3 and 5 days and had an average of 4.17 days. The average for the total 111 cycles was 3.9 days. Some of the indi-

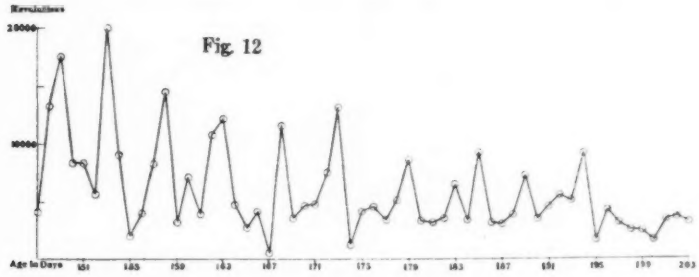


Fig. 12

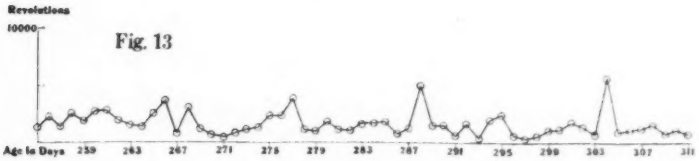


Fig. 13

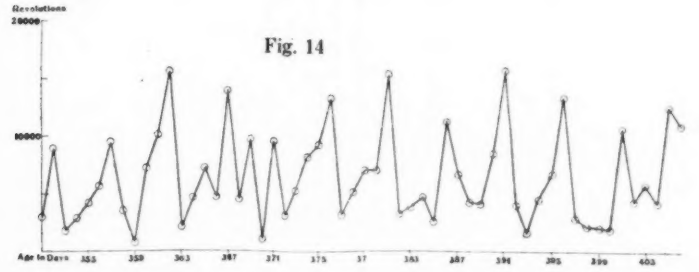


Fig. 14

Fig. 12. Curve of activity of rat 3—1918 from the age of 147 days to 203 days showing the effect of an inadequate diet on the oestrual cycles and amount of activity.

Fig. 13. Curve of activity of rat 3—1918 from the age of 255 days to 311 days, showing the effect of an inadequate diet on oestruation and amount of activity. During this time oestrum occurred at the ages of 266, 277, 288, and 304 days.

Fig. 14. Curve of activity of rat 3—1918 from the age of 351 days to 406 showing recovery of normal cycles after the administration of an adequate diet.

viduals, notably 3, 5, 6, 8, D1, D4 and D5, showed rather marked uniformity in the length of their cycles, while others showed great individual variation. No cycle, however, was of greater than 7 days' duration. The irregularity in the length of cycles was found more prevalent in very young and in old animals than in those in the prime of life. Of these 111 cycles there were 12 of 2 days' duration, 21 of 3 days, 57 of 4 days, 16 of 5 days, 2 of 6 days, and 3 of 7 days. The period of 4 days occurred

TABLE 1

Showing the age of first oestrum and length of successive cycles in young rats before the average age of 84 days

RAT	AGE OF FIRST OESTRUM	AGE AT LAST OBSERVED OESTRUM	LENGTH OF CYCLES IN DAYS
1A	73	90	5, 4, 3, 5,
2	81	90	5, 4,
3	81	89	4, 4,
5	77	79	4, 4, 4,
6	83	91	4, 4,
7	77	92	4, 4, 3, 4,
8	59	90	5, 4, 4, 4, 4,
D1	61	80	4, 4, 4, 4, 3,
D2	51	82	4, 2, 2, 4, 4, 4, 4, 3, 4,
D3	61	82	4, 2, 4, 3, 3, 5,
D4	46	79	4, 4, 5, 4, 4, 4, 4, 4,
D5	67	83	4, 4, 4,
E1	55	83	3, 5, 3, 2, 3, 4, 4, 4,
E2	59	83	4, 5, 3, 4, 2, 2, 4,
E3	61	83	7, 3, 4, 3, 5,
E4	67	88	4, 4, 2, 7, 4,
E5	63	88	4, 2, 2, 3, 3, 4, 3, 4,
F1	55	79	5, 3, 4, 4, 3, 5,
F2	66	78	6, 2, 4,
F3	52	77	3, 2, 5, 4, 5, 3, 3,
F4	55	79	5, 5, 2, 4, 3, 5,
F5	59	76	7, 6, 4,
Average.....	64	83.7	First cycle 4.5 days. Last cycle 4.17 days. Total cycles 111. Average cycle 3.9 days.

a little more than half the times, the sum of all the other periods being 54. The ratio of 3-day periods to 5-day periods is approximately 7 to 5.

Table 2 shows the length of successive cycles of the individual rats from puberty to the age indicated. This latter age was about the prime of sexual life. Several mating attempts and other tests were made on these animals which resulted in disturbing the normal oestrual sequence. The numbers in parentheses indicate the days which intervened after such trials during which no oestrum occurred. Some of these

TABLE 2

Showing age of first oestrum and the length of successive cycles between the ages indicated in young females. Numbers in () indicate mating trials, etc.

RAT	AGE AT FIRST OESTRUM	AGE AT LAST OBSERVED OESTRUM	DAYS DEDUCTED ACCOUNT MATING TRIALS	LENGTH OF CYCLES IN DAYS
1A	73	139		5, 4, 3, 5, 3, 3, 6, 4, 7, 3, 4, 9, 3, 4, 4
1B	118*	251	24	4, 4, 4, 4, 4, 4, 4, 4, 4, 5, 4, 4, 5, 6, 4, 5, 4, 4, (9), 5, 4, (15), 4, 5, 4, 4, 4, 4, 3, 5, 4, 4
2	81	318	32	5, 4, 4, 3, 5, 4, 4, 3, 5, 3, 5, 3, 2, 3, 4, 7, 5, 4, 4, 4, 4, 4, 4, (13), 4, 4, 4, 4, 4, 5, 4, 4, (8), 3, 5, 4, 4, (11), 4, 4, 4, 2, 4, 4, 4, 4, 4, 4, 4, 3, 4, 4, 4, 4
3	81	317	66	4, 4, 5, 3, 4, 4, 2, 2, 4, 4, 4, 3, 5, 2, 6, 4, 4, 5, 3, 4, 4, 4, 4, 4, 4, 4, 4, 4, 3, (27), 5, 4, 4, 4, 4, 4, 5, 4, (24), 4, 4, 4, (15), 3, 4, 5
4	109	317	46	4, 4, 5, 5, 3, 4, 4, 3, 5, 4, 3, 5, 4, 3, 4, 5, (6) 4, 3, 5, 4, 4, 4, 4, 4, 3, 5, 4, (13), 4, 3, 3, 5, 4, (13), 4, 4, (14), 4, 4, 4, 4, 4, 4, 4
5	77	318	68	4, 4, 4, 2, 2, 3, 5, 2, 2, 4, 4, 3, 3, 2, 3, 3, 2, 2, 6, 4, 4, 4, 4, 4, 4, 4, 4, 3, 5, 4, 4, 4, (26), 5, 5, 6, 4, (21), 4, 4, 4, 4, 4, (21), 4, 4, 2, 2, 4, 4
6	83	296	24	4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 3, 5, 3, (9), 2, 4, 4, 4, 4, 3, 8, 4, 3, 5, 5, (15), 4, 4, 5, 4, 4, 5
7	77	294	21	4, 4, 3, 4, 2, 4, 5, 3, 3, 2, 4, 3, 5, 4. 3, 3, 5, 3, 5, 4, 4, 3, 5, 3, (6), 3, 5, 4, 2, 2, 3, 2, 5, 3, 4, 6, 4, 3, 10, 10, 3, 4, 3, 4, (15), 4, 4, 4, 3, 5, 4
8	59	294	57	5, 4, 4, 4, 4, 4, 4, 4, 4, 4, (8), 4, 6, 3, 5, 4, 4, 5, 4, 3, 5, 3, 5, 4, 4, 4, (15), 4, 4, 4, 4, (9), 4, 4, 4, 5, 4, 4, (7), 4, 4, 4, 4, 4, 4,4, 4, 4, (18)

* 1A died at age of 140 days. The first observed oestrum of 1B after replacing 1A —had been under observation only 8 days.

represent pregnancy, others pseudo-pregnancy, etc., which will be discussed in a later paper. It will be noticed that the length of these disturbances approached very closely to some multiple of 4.

A total of 368 oestrual cycles is recorded in this table. Of this number 225 conformed to a 4-day period. This number is over one and one-half times greater than the sum of all the other cycles. The next in numerical value were 3- and 5-day periods, each of which occurred 55 times. A period of 2 days was found 21 times; 6 days, 6 times; the remaining 7-, 8-, 9- and 10-day periods happened 2, 1, 1 and 2 times respectively. It is interesting to note that in those observations the periods of 3 and 5 days occurred in sequence 28 times. Since their sum is a multiple of four, the significance of this arrangement may be more than coincidence. The ratio of 3-day periods to 5-day periods was as 1 to 1.

At the close of the observations comprising table 2, all the animals were removed from the revolving cages and each placed in a separate stationary cage with a strong and vigorous male. This was done as an absolute test for sterility. All the females threw one or more litters during the testing period, which lasted approximately 100 days. During this time no observations were made relative to the oestrual cycles.

Table 3 gives the length of successive oestrual cycles of the same rats as table 2, after they had been returned to the revolving cages. It represents the sequence from the average age of 417 days to the menopause at the average age of 755 days. During this entire time they were not disturbed by mating trials or other tests. A comparison of this table with table 2 shows that the length of the cycles had become much more irregular. In the younger rats there were only two periods which required two digits to express their length (10 days each) while in this case there were 35 periods, ranging from 10 to 45 days in length. In general these longer periods were found toward the end of sexual life. Each of the long quiescent periods of 21, 31, 35 and 45 days occurred but once.

This table also shows the relation of the cycles to the menopause and emphasizes our statement that no two of these animals approached the end of sexual life in the same manner. Rat 1B, with the exception of four abnormally long periods of dioestrum, had been showing fairly regular oestrual cycles until near the end when oestrum ceased for 21 days. Had observation stopped during this time one would have concluded that the menopause had taken place with the last preceding oestrum. However, at the end of this period of apparent rest, oestruation was reëstablished for five regular 4-day periods after which it stopped completely. Rats 4 and 5 showed a much different type. A single oestrum occurred to be followed by a long period of rest. This was repeated six times in no. 4. In rat 8 a still different type was observed. Thirty-five days of rest elapsed before the last single oestrum which marked the menopause.

There are recorded in this table 493 cycles. The regular 4-day period, which was so prevalent in young animals, was found but 206 times. This

is considerably less than one-half of the total observed cycles. The 3- and 5-day periods were found 61 and 94 times respectively. This forms

TABLE 3

Showing the length of successive oestrual cycles of the same animals as table 2 during approximately the last half of their sexual life

RAT	AGE AT BEGINNING OBSERVATION	AGE AT MENOPAUSE	LENGTH OF CYCLES IN DAYS
1B	387	735	6, 3, 5, 4, 4, 4, 4, 4, 5, 3, 4, 3, 10, 4, 4, 5, 5, 13, 12, 4, 5, 4, 4, 4, 3, 4, 10, 3, 5, 6, 7, 8, 4, 3, 4, 3, 4, 8, 4, 8, 7, 8, 2, 5, 4, 4, 4, 5, 14, 4, 4, 4, 4, 4, 4, 5, 3, 3, 6, 3, 3, 21, 4, 4, 4, 4, 4
2	423	778	5, 4, 4, 4, 4, 4, 4, 5, 5, 3, 4, 4, 4, 4, 4, 6, 3, 4, 4, 4, 5, 4, 4, 4, 3, 5, 10, 4, 2, 5, 5, 5, 5, 5, 6, 4, 3, 6, 5, 5, 3, 2, 5, 3, 5, 6, 5, 2, 4, 8, 2, 3, 6, 4, 6, 18, 5, 4, 9, 19, 5, 4, 7, 3, 13, 6, 5, 8, 5
3	425	768	4, 4, 4, 6, 4, 7, 5, 3, 4, 5, 4, 15, 5, 4, 4, 3, 5, 4, 4, 4, 3, 5, 6, 4, 4, 4, 9, 5, 6, 16, 5, 8, 4, 15, 3, 6, 5, 4, 7, 5, 19, 19, 4, 4, 4, 5, 11, 4, 12, 9, 14, 5, 5, 4
4	426	760	4, 4, 4, 4, 5, 4, 4, 4, 4, 4, 4, 3, 3, 3, 4, 4, 4, 4, 5, 3, 7, 4, 3, 4, 5, 5, 9, 5, 4, 4, 4, 8, 8, 9, 4, 5, 9, 4, 4, 4, 4, 4, 4, 5, 5, 10, 5, 6, 4, 12, 18, 14, 5, 18, 15, 17
5	429	782	4, 4, 5, 3, 5, 4, 4, 4, 4, 4, 4, 4, 5, 2, 5, 5, 3, 4, 4, 4, 4, 4, 4, 4, 3, 5, 4, 7, 4, 4, 4, 4, 5, 5, 4, 3, 3, 3, 2, 4, 4, 4, 3, 5, 4, 3, 4, 4, 4, 4, 4, 3, 4, 4, 3, 5, 5, 4, 5, 7, 8, 13, 9, 11, 3, 9, 4, 14, 5, 11, 12, 10
6	402	741	7, 4, 4, 4, 4, 3, 5, 4, 5, 4, 3, 5, 4, 4, 3, 4, 6, 4, 4, 8, 4, 4, 5, 4, 4, 5, 6, 4, 5, 3, 5, 5, 3, 3, 4, 4, 6, 3, 4, 4, 6, 4, 3, 4, 4, 4, 3, 4, 4, 4, 4, 5, 45, 4, 4, 4, 5, 12, 18, 12, 5, 5
7	403	716	4, 4, 6, 3, 4, 2, 6, 6, 3, 7, 4, 2, 5, 6, 4, 2, 14, 6, 9, 5, 3, 6, 4, 4, 2, 6, 3, 5, 3, 6, 5, 4, 2, 2, 6, 6, 7, 5, 4, 4, 3, 5, 3, 5, 4, 4, 11, 9, 5, 4, 5, 6, 15, 30, 4, 11
8	441	761	6, 13, 4, 4, 4, 4, 3, 5, 4, 4, 4, 4, 4, 5, 3, 4, 4, 4, 5, 4, 3, 5, 4, 4, 4, 7, 6, 4, 4, 4, 4, 5, 7, 12, 4, 5, 2, 4, 3, 8, 5, 4, 8, 5, 14, 6, 5, 4, 6, 9, 3, 6, 7, 4, 6, 35

a ratio 1 to 1.54. In other words, there was a relative shifting of the 3- and 5-day cycles from young to old age conditions.

A condensed summary of tables 2 and 3 is given in table 4. From this a direct comparison of young and old rats is readily made, not only

of the same individuals but also of the averages. As will be noticed the days of cessation of oestrum, due to mating trials, etc., have been deducted. In the young rats it will be noticed that the average length of the cycle, as exhibited by the different individuals, varied from 3.52 to 4.46 days, and that the average for the group was approximately 4 days. During the last part of their sexual life these same rats showed an average variation from 4.90 to 5.70 days and an average of 5.53 days for the group. The individual average for the whole sexual life span and the computed number of cycles that would have occurred in each rat during its entire

TABLE 4

Showing in days the age of the first oestrum and menopause, the duration of sexual life and the effect of age on the length of the oestrual cycle

	YOUNG FEMALES, FROM FIRST OESTRUM TO AGE INDICATED						OLD FEMALES, FROM AGE INDICATED TO MENOPAUSE							
No. of rat	Age at first oestrum	Age at last observed oestrum	Number of days observed	Days deducted account mating trials	Number of cycles	Average interval	Age at first observed oestrum	Age at menopause	Number of days observed	Number of cycles	Average cycle	Duration of sexual life	Total average cycle	Computed number of total cycles
1A	73	139	67		15	4.46								
1B	118*	251	109	24	31	3.52	387	735	348	68	5.12	654	4.60	142
2	81	318	205	32	52	3.94	423	778	355	69	5.15	696	4.62	150
3	81	317	170	66	43	3.95	425	768	343	54	6.35	687	5.28	130
4	109	317	162	46	40	4.02	426	760	334	56	5.95	651	5.17	126
5	77	318	173	68	47	3.70	429	782	353	72	4.90	705	4.42	159
6	83	296	189	24	46	4.10	402	741	339	62	5.47	658	4.89	141
7	77	294	202	21	49	4.12	403	716	313	56	5.60	639	4.90	130
8	64	294	186	57	45	4.13	441	761	320	56	5.70	697	5.00	139
Total.	645	2544	1463	332	368	35.94	3336	6041	2705	493	44.24	5387	38.88	1117
Ave..	80.6	282	183	41.5	46	3.99	417	755	338	61.6	5.53	673	4.86	140

*1 A died and was replaced by 1B. 118 days was the first oestrum observed after putting in revolving cage, eight days previously and does not mean its first oestrum.

sexual life, if it had not been disturbed, are given in the last two columns of the table. It is noticed that the individual variation in total average cycles ranged from 4.42 to 5.28 days and the possible number of total cycles varied from 126 to 159 times. The total average length of cycles for the group was 4.86 days and the total average number of computed cycles was 140.

It is thus seen that in speaking of the length of the oestrual cycle in rats the age of the animal must be taken into consideration. The work of Long and Evans (4) dealt with young animals. The 54 rats whose

ages I have gotten ranged from 37 to 284 days of age during their observations and the average for the group was from 100 to 170 days. Wang (6) also dealt with young animals whose ages at the beginning of his experiments were: Group 1, 60 days; group 2, 70 days; group 3, 30 days; and group 4, 20 days. Our results cannot therefore conform wholly to theirs. The first of these investigations gives the length of the cycle as about 4 days. The sum of the lengths of their five stages of the cycle is 4 days

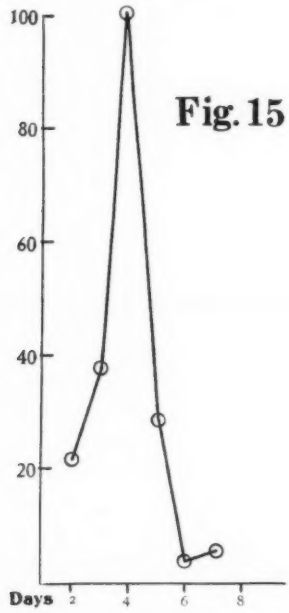


Fig. 15

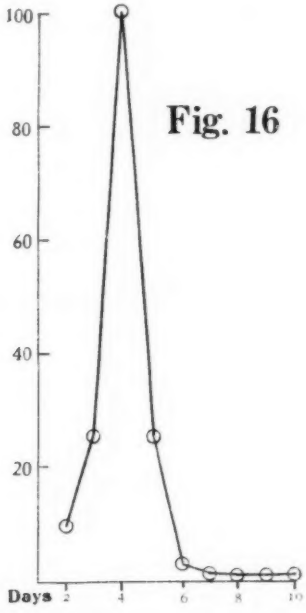


Fig. 16

Fig. 15. Curve of frequence of cycles of different lengths in young rats from the age of puberty to about 80 days. It shows the relation of 100 periods of 4 days duration to other periods observed during this time.

Fig. 16. Curve of frequency of oestrual cycles of different lengths, giving the relation of 100 cycles of 4 days to other length cycles, in rats from the age of pubescence to the average age of 283 days.

and 6 hours. The latter investigator gives 4.3 days as the length of the activity curve which corresponds to an oestrual cycle. Our results correspond closely for animals of the same age, but show an average lengthening of the cycle with advancing age.

The results of tables 1, 2 and 3 are put in graphic form in figures 15, 16 and 17 respectively. For the sake of ready comparison the number of times different periods were observed were converted into reciprocal

numbers in which the number of times 4 days occurred was equal to 100. The ordinates thus represent the number of times the periods occurred and the abscissa the length of cycles in days. The number of times each period occurred is thus represented in relation to the normal 4-day periods. Thus in figure 15 if there had been 100 periods of 4 days there would have been a little over 20 of 2 days, a few less than 40 of 3 days, and about 30 of 5 days, etc.

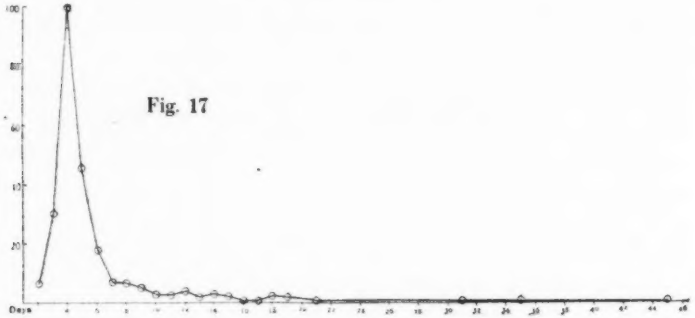


Fig. 17

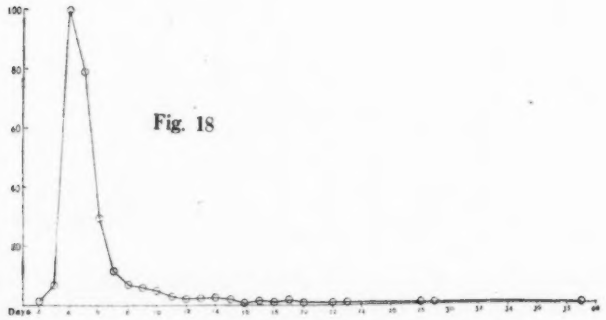
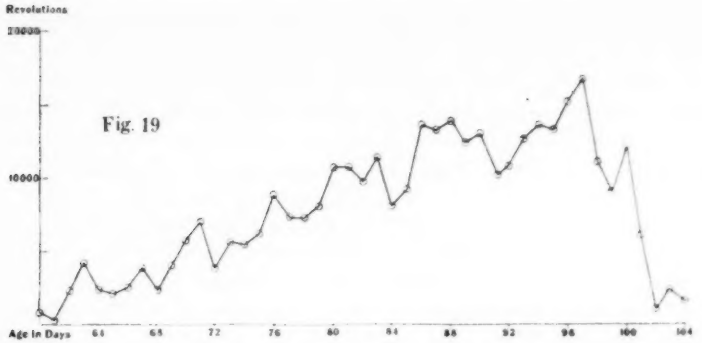


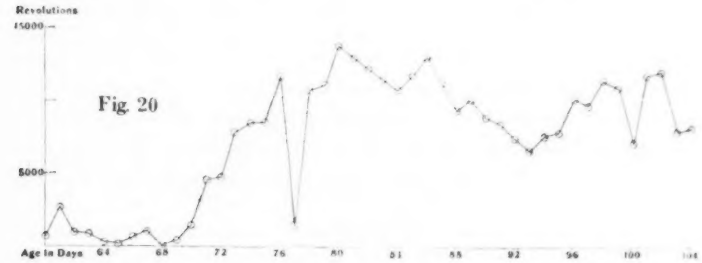
Fig. 18

Fig. 17. Curve of frequency of the different lengths of oestrual cycles of rats from the average age of 417 days to the menopause, giving the relation of 100 periods of 4 days in length to other periods.

Fig. 18. Curve of frequency of different length cycles recorded by Long and Evans (4) on rats between the average ages of 100 and 170 days, the relation of 100 periods of 4 days duration to other periods is given.

Comparing figure 16 with figure 15, we notice that the number of 5-day periods with reference to the periods of 4 days was almost the same, but that the 2- and 3-day cycles had become much fewer in number. Also that the curve has been extended to the right due to the occurrence of longer periods. The whole effect is that of a slight increase in the length of the average cycles.





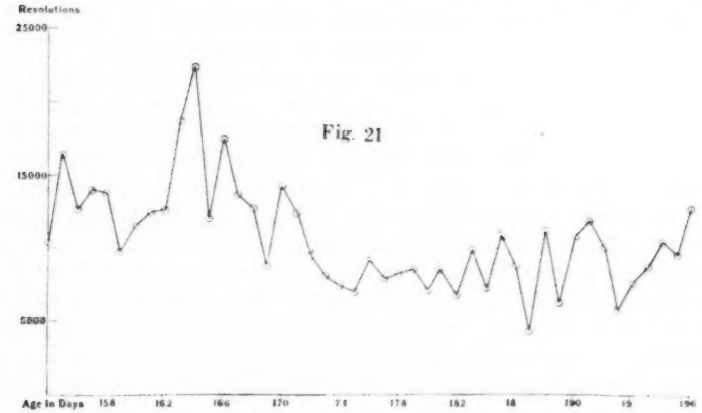


Fig. 19. Curve of activity of male rat A2 from the age of 60 days to 104 days, showing absence of regular rhythmic spurts of activity similar to female records. An increase in amount of activity after pubescence is shown similar to that exhibited by the females.

Fig. 20. Curve of activity of male rat A3, a brother of A2 (fig. 19), from the age of 60 days to 104 days.

Fig. 21. Curve of activity of male rat A4 from the age of 154 days to 196 days showing the absence of a regular rhythm.

Examination of figure 17, which represents the frequency curve of the length of the cycles during the last half of sexual life, shows that there is a further relative lowering of 2-day periods, a marked increase in the 5- and 6-day cycles and a great extension of the curve to the right, all of which indicates that there was a further lengthening of the cycles.

In order to make a more direct comparison of the results of Long and Evans in regard to the length of the oestrual cycles we have constructed a frequency curve from the data given in their table 37. This is shown in figure 18 which is constructed from the 1373 cycles they record. Since this table represents young animals whose ages averaged 100 to 170 days this figure should be compared to one which would represent conditions between that of figure 15 and figure 16. But comparing figure 16 with figure 18 we note a marked difference in the relation of the 2-, 3-, 5-, 6- and 7-day cycles to the 4-day periods and the occurrence of many long periods. They found relatively fewer 2- and 3-day cycles and a relatively great increase in 5-, 6- and 7-day cycles. This may have been partly due to the mixed breed of rats which they used (a cross between the albino and the gray rat), since it has been shown that the gray rat has a cycle of 5 days. Another factor which may have had its influence was their method of determining oestrum by vaginal smears. The number of long periods of dioestrum which they found in their young animals agrees with our results. We have found that the method of vaginal smear tests, due to necessary handling of the animals, etc., has a retarding influence on oestrum and disturbs the normal sequence.

ACTIVITY OF THE MALES. In the early part of this paper we stated that a rhythm of activity similar to that of the females was not found in the males. In order to substantiate this statement we have constructed the activity curves of three male rats, A2, A3 and A4. These are shown in figures 19, 20 and 21 respectively. A1 and A2 were brothers whose ages were 60 days at the beginning of the figures. This age is slightly earlier than the average age of puberty. Figure 21 represents an older animal about in its prime. Spurts of activity are seen in each of these curves but they do not occur in regular sequence. That is, no periods of uniform rhythm are manifested. The cause of these fluctuations has not yet been determined. Figures 19 and 20 show that young males, after reaching the age of puberty, exhibited a gradual average increase in daily activity very similar to young females.

SUMMARY

From the above discussion we may deduce the following conclusions:

1. The isolated female rats exhibited marked rhythmic increases in voluntary activity during their sexual life. This rhythm was not manifested prior to pubescence nor after the menopause.

2. The males did not show any rhythmic fluctuations in activity.

3. The peak of activity corresponded to the cornified cell stage and indicated oestrum. The female readily accepted the male at that time.

4. The time from one peak to the next indicates the duration of an oestrual cycle.

5. The fluctuations in activity were greatest at the age of about 120 days. They became less and less until the menopause.

6. The oestrual cycle was more regular in young animals than in old ones.

7. The oestrual cycle was shorter in young than in old rats. In young up to the average age of 283 days it averaged approximately 4 days. In old animals from the average age of 417 days to the menopause it averaged 5.53 days. From pubescence to the menopause the average was 4.86 days.

8. The determination of oestrum by vaginal smear tests tended to disturb the regular sequence of oestrum and to lengthen the period of dioestrum.

9. The average age of pubescence and the menopause of our females was 80.6 days and 755 days respectively.

10. The average duration of sexual life of our female rats was 673 days during which 140 oestrual cycles were possible.

BIBLIOGRAPHY

(1) Slonaker: Journ. Animal Behavior, 1912, ii, 20.
(2) Slonaker: Anat. Rec., 1908, ii, 116.
(3) Slonaker: Science, 1923, lvii, 180.
(4) Long and Evans: Mem. Univ. of Calif., 1922, vi.
(5) Miller: Amer. Nat., 1911, xlv, 623.
(6) Wang: Comp. Psychol. Monographs, 1923, ii, September.
(7) Goetsch: Bull. Johns Hopkins Hosp., 1916, xxvii, 29.
(8) Frank: Journ. Amer. Med. Assoc., 1919, lxxiii, 1764.
(9) Sisson and Broyles: Bull. Johns Hopkins Hosp., 1921, xxxii, 23.
(10) Marshall: The physiology of reproduction, 1922.
(11) Halban: Sitzungsb. Akad. Wissensch. Wien, 1901, cx, Abth. iii, 71.
(12) Knauer: Arch. f. Gynäk., 1900, lx, 322.
(13) Marshall and Jolly: Trans. Roy. Soc. Edin., 1907, xlv, 589.
(14) Fraenkel: Arch. f. Gynäk., 1903, lxviii, 438.
(15) Marshall and Runciman: Journ. Physiol., 1914, xlix, 17.
(16) Frank: Journ. Amer. Med. Assoc., 1922, lxxviii, 181.
(17) Allen and Doisy: Journ. Amer. Med. Assoc., 1923, lxxxi, 819.
(18) Slonaker and Card: This Journal, 1923, lxiv, 167.
(19) Evans and Bishop: Journ. Amer. Med. Assoc., 1923, lxxxi, 889.

THE PHYSIOLOGY OF THE ATRIO-VENTRICULAR CONNECTION

IV. Functional Differentiation and Innervation of the A-V Connection in the Alligator Heart[1]

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A functional differentiation in the A-V connection of turtles and lizards was demonstrated by Laurens (1a), (1b) in that the right and left portions of the funnel were found to be most effective in conducting the impulses from auricle to ventricle. The ventral portion immediately contiguous to the sides, particularly on the left, was sometimes found to be important. Even when reduced to very narrow threads of tissue these portions were still capable of preserving coördination between A and V. The basis for this physiological differentiation was shown by histological investigation (2a), (2b) to be a more intimate connection between these parts of the funnel and the ventricle. In a later paper (3) this functional differentiation was extended to irritability and impulse formation, the demonstration being made that these characteristics were more highly developed in the lateral portions of the funnel than elsewhere.

As every one knows, Gaskell many years ago investigated this question in the tortoise but not in a thorough or detailed manner. He reported that the portion of the atrio-ventricular junction which was mainly responsible for coördination between the auricles and the ventricle was ventral and close against the aorta, and that the lateral part near this was partly responsible and the dorsal not at all. Gaskell (4, p. 104) states that Laurens obtained exactly the same results as he did, which it is necessary to say does not agree with the facts set forth in the articles above referred to. He further writes that Laurens found no muscular continuity from auricle to ventricle on the dorsal side, which is quite incorrect, as any one can see by glancing over Laurens' two articles dealing with the structure of the A-V connection. Barry (5) has recently reported that the left ventral part of the A-V connection is the most important

[1] The data are taken from the essay presented by Edith C. Lowman for the degree of Master of Science, Yale University, 1923.

for conduction from A to V; and Eyster and Meek (6, p. 29) believe that the ventral portion is most important for coördinated conduction, from the point of view that it is the first part to show the negative wave as the impulse passes to the ventricle.

Haberlandt (7) has also demonstrated a functional differentiation in the turtle in that the sides of the connection, and particularly the left, when stimulated, have a higher ability to develop automatic contraction in the still heart than the dorsal and ventral portions have.

One finds statements in the literature (8), (9) to the effect that in the lizards and turtles the nervous and muscular portions of the A-V connection are separate and can be so experimentally dealt with. Such is far from the case. As pointed out by Laurens and others, the dorsal ligament, with its coronary nerve or nerves, can be easily dispensed with as far as A-V conduction or coördination is concerned. Haberlandt (7a) has recently demonstrated the same thing.

Gaskell (4, p. 103) speaks of a flattened band of tissue on the dorsal side of the heart between the sinus and the ventricle. From this band the reticulated structure of each auricle arises. He calls this flattened band the junction wall between the auricles, and states that its muscles are much more circularly arranged than are those of the bulged portion of the auricles. Later (p. 104) he says that what he calls the junction wall between the two auricles is what Laurens designates as the dorsal ligament. This is not absolutely correct, because Laurens did not find muscle tissue in the dorsal ligament, and according to Gaskell it does occur in the junction wall. Gaskell is probably describing something quite different from what Laurens calls the dorsal ligament.

The A-V connection in lizards and turtles, as well as in frogs, is composed of muscle and nerve so intimately connected and intermingled that it may be impossible to put one out of action without at the same time injuring the other. Claims that the results obtained after cutting or injuring parts of the A-V funnel in such animals support the myogenic or neurogenic theory of conduction and coördination in the heart are based on insufficient premises.

An investigation on the alligator similar to that carried out on the A-V connection of the turtle and lizard has been under consideration for some time. The heart of the alligator is very favorable for experimentation of this kind, being large enough in small animals to work with conveniently. The dorsal ligament is surprisingly large for the size of the heart and the A-V junction is easily accessible to section. It is well known that the heart of all reptiles, except the crocodilia, is possessed of a single ventricle and that the cavity of this ventricle is incompletely subdivided by an incipient septum which is completed in the crocodilia. The four-chambered heart of the latter is essentially like that of birds and turtles in that the

aerated blood is on the left side of the septum (complete or not) contrary to other reptiles in which the non-aerated blood is on the left side.

A description of the structure and relations of the heart of the alligator with special reference to the A-V connection and dorsal ligament has just been published by Swett (10). He finds the general structure of the dorsal ligament in the alligator to be similar to that described for the tortoise by Laurens. It consists of a more or less cylindrical fold of the pericardium which separates itself from the wall of the sinus about the middle of its ventral surface and passes ventro-cephalad to the auriculo-ventricular groove, running free over the dorsal surface of the right auricle. At its points of connection with the sinus and ventricle it broadens slightly into a fan-shaped structure, becoming immediately indistinguishable from the surrounding pericardium. Examination of this structure in the living heart reveals the fact that it transmits the coronary vein from the auriculo-ventricular groove to the sinus. Histological study readily shows that, in addition to the coronary vein, there are included in this ligament a more or less variable number of nerve branches and a small artery. The areolar connective tissue in which the nerves and blood vessels are imbedded is similar to and contiguous with that under the pericardium in all regions. Ganglion cells in profusion are also contained in the ligament as elsewhere under the pericardium, but no muscle fibers are present.

Swett finds that in the alligator the interauricular septum is directly continuous with the interventricular. Its muscular portion goes over into the muscular part of the interventricular septum posteriorly and its membranous caudal border is continuous with the adjacent part of the interventricular septum. In the alligator the auriculo-ventricular bundle has not undergone such marked reduction as is described by Mackenzie (11) for the crocodile. There is a considerable invagination of auricular fibers into the lateral wall of the right ventricle and into the posterior part of the interventricular septum, while in the left ventricle the bundle, though not so strong as in the right, nevertheless is present as a definite though interrupted sheet of fibers. The connection in the alligator differs from the condition found in the hearts of the lower reptiles, in not being a complete funnel or ring on account of the interposition of the membranous bulbus on the ventral side. A narrow tongue-like band projects ventrally into the interventricular septum from the posterior wall of the partial funnel, giving the bundle somewhat the appearance of an "M" in optical cross-section. The sheet into the right ventricle is quite strong and descends for a considerable distance before merging with the ventricular musculature. In the left ventricle, at the base of the parietal valve, the sheet of descending auricular fibers is interrupted by connective tissue and even between the valves it does not extend far into the ventricle.

In its finer structure the connecting tissue resembles the musculature of the auricles very closely. At the region of the auriculo-ventricular openings the fibers are arranged in a more or less circular fashion, but lower down in the ventricle they become directed longitudinally. The distribution to and the intermingling with the ventricular fibers is easily observed, inasmuch as the striations of the latter are much wider and take the stain more deeply. No nerve fibers or ganglion cells could be found in the bundle, although many were seen in the auriculo-ventricular groove and traced to points in the connective tissue very close to the conducting bundle.

This anatomical study was a necessary introduction to the investigation as to whether a functional differentiation in the atrio-ventricular connection of the alligator could be demonstrated. The results of this study are given in the following pages. Observations on the nervous control of the A-V connection in normal hearts as well as in those in which block had been induced were also made.

The experiments were performed on young alligators, 18 to 24 inches long. The brain and spinal cord were destroyed with as little loss of blood as possible. The heart was exposed and its rate counted before the pericardium was opened. The pericardium was then slit, and the heart *in situ* suspended for graphic registration from the right auricle and the apex of the ventricle.

To test the functional differentiation of the A-V connection small parts of it were cut through with fine scissors, a record being taken after each cut to determine the effects on the A-V interval and on the ventricular contraction. Sufficient time was allowed to elapse after each cut, before cutting further, for the heart to recover from any temporary disturbance as well as for improvement in the degree of block which the specific cutting had occasioned. The A-V ring is considered as divided into eight parts as follows: dorsal; dorso-lateral, right and left; left lateral; right lateral; ventral; ventro-lateral, right and left.

Before cutting the dorsal ligament, it was double ligated in order that both proximal and distal ends could be electrically stimulated after section. The needle electrodes were placed as close together as possible and care was taken that the chance of escape of current to other parts of the heart was reduced to a minimum.

The dorsal ligament. Cutting the dorsal ligament, including the coronary nerve, has merely temporary effects of either excitation or inhibition, the rate and A-V interval being increased or decreased.

In the majority of cases when the distal, or sinus, end of the cut coronary nerve is excited by an interrupted current typical inhibitory effects are exerted upon the auricles, slight with moderate strengths of current, and greater up to complete stoppage with stronger. In about 80 per cent

of the cases the inhibition, even with strong currents, is on the rate alone, while in the remainder the amplitude of auricular beat as well as its rate is affected. The A-V interval usually remains unchanged, indicating no apparent impairment of conduction in the A-V connection. The above inhibitory effects were obtained not only on hearts exhibiting normal A-V rhythm but also on those in which partial block had been artificially produced. No effects whatsoever were evident in the mechanical registration following stimulation of the proximal, or ventricular, end of the coronary nerve.

These results indicate that the dorsal ligament in the alligator heart, as in the turtle, plays no indispensable part as a conductor or coördinator between the A and the V. There is no evidence to support Veil's (12) statement that there is functional identity between the sino-ventricular connection in lower vertebrates and the bundle of His in mammals. Coördinated A-V conduction in the alligator, as in the turtle and lizard, is not interfered with by its section, the slight effects observed being referable to mechanical stimulation incident to cutting it.

In some cases an inhibitory effect upon the auriculo-ventricular connection was demonstrated in consequence of the stimulation of the distal end of the coronary nerve. This was particularly observed after partial block had been experimentally induced. A typical case may be cited by way of illustration. In this case of block, one of dropped beats, the A-V interval had successive values of 0.5, 0.75 and 0.8 second, after which a beat was dropped. Then another series with A-V intervals of 0.4, 0.6 and 0.8 second, again followed by a dropped beat. At this point the stimulation was commenced, the A-V intervals being 0.5, 0.75 and 0.8 second, followed by a dropped beat, then one of 0.5 second and again a dropped beat. The stimulation was now stopped. The next A-V interval was 0.4 second long, after which a beat was dropped and the heart continued to exhibit a 2:1 rhythm for some time with an A-V interval of 0.4 second. Haberlandt (7a) has demonstrated some interesting results on stimulating the coronary nerve in the turtle. He finds when the dorsal ligament (coronary nerve) and A-V funnel are simultaneously stimulated with faradic shocks, that flutter and fibrillation of the auricles and ventricles of greater or less duration may be induced. The effect, however, was much slighter than that obtained by stimulating the vagus itself, together with the A-V funnel. The stimulation of the coronary nerve alone usually produced negative inotropic and chronotropic effects; but in some cases the strongest stimulus had very slight if any effect. In a few cases stimulating the coronary nerve alone caused the A and the V to flutter and fibrillate. Stimulation of the coronary nerve during a previously induced flutter had but slight effect, but did exert a completely inhibitory influence on automatic contractions of the

V following previous flutter. Cutting the dorsal ligament had no influence on the fibrillating V. In one case stimulation of the coronary nerve increased the tonic contraction of the A with concomitant decrease in the height of the clonic contractions.

In further experiments Haberlandt (13) demonstrated on the normal beating heart a negative chronotropic influence following stimulation of the coronary nerve, which was not sectioned. This was also demonstrable when the central end of the cut nerve was stimulated; but not so when the peripheral end was stimulated, there being only an inotropic effect under these conditions. In a few experiments he found that stimulation of the peripheral end of the coronary nerve was followed by a few ventricular contractions, arising from the ventricular part of the A-V funnel, and immediately followed by auricular contractions.

The results of sectioning the various portions of the A-V connection. Dorsal and right dorsolateral portions. As in the turtle and lizard, the dorsal part of the connection can be cut away with little effect, other than a slight temporary change in rate and A-V interval. When these two portions are the only connection between A and V complete dissociation results, the V usually remaining still.

It should be mentioned that there is always a total cessation of beats for an interval after any part of the connection is cut. The duration of the ventricular standstill varies with the part of the connection remaining but the shortest standstill follows the severing of the dorsal and right dorsolateral connection.

Right lateral portion. Cutting this part of the connection produces a ventricular standstill of, on the average, eleven seconds. When A-V rhythm is regained, the A-V interval is markedly increased (1.9 second) from which high value it gradually decreases.

Partial block is produced by cutting this part of the connection through at the level of the A-V groove. The following notes indicate the course of a typical case: 3:2 and 2:1 rhythm alternately for nine minutes followed by one cycle of 4:3 and one of 3:2 rhythm, then three ventricular systoles dropped, after which a normal 1:1 rhythm appeared. When this part of the connection which is anatomically the most obvious portion of the bundle, is cut through below the level of the A-V groove in the upper third of the V, complete block results. Cutting the ventricle through at this level in other portions does not produce this result. In agreement with the histological findings, this portion is functionally very important for coördinated A-V rhythm. When the connection is cut through at higher levels, the impulse for coördinated contraction is still capable of passing by other means from A to V. In other words, while the impulse may be conducted through the base of the ventricle by differentiated tissue other than the right lateral part of the connection, severing this

portion at a lower level so as to remove all other possible connections of the A with this portion of the A-V connection proper destroys all conducting pathways between the upper third and lower two-thirds of the ventricle. Nevertheless, when it is the sole connection it is incapable of preserving perfect A-V coördination.

Right ventrolateral and left dorsolateral portions. Partial block (dropped beats) is sometimes produced by cutting either of these. The recovery from block is usually complete although the A-V interval is usually much lengthened.

Ventral portion. In only one case was a 1:1 rhythm regained within several minutes after cutting this portion of the connection. In seven cases the disturbance in conductivity varied from complete dissociation, when other parts in addition had been cut, to slight partial block. Improvement of the block may continue until only every tenth or eleventh ventricular systole is missed, although the A-V interval is always considerably lengthened, and complete recovery never takes place. The most vital part of the ventral portion is that bordering on the left ventrolateral. When this is sectioned complete block usually results, partial block always. It requires either the left ventrolateral or left lateral part as well in order that coördination may exist.

Left lateral portion. All degrees of partial block are obtained when this is cut. In some cases there is a decidedly irregular ventricular rhythm, with alternate long and short A-V intervals. In others, after ventricular standstill of several minutes, partial block (dropped beats) occurs. The block improves but never recovers completely, remaining at 10:9 or 12:11 rhythm, indicating that the fibers of this part of the connection are important for perfect coördination.

Left ventrolateral portion. Cutting this through occasions, after a ventricular standstill of from two to seven minutes, a moderate block, such as 5:4 rhythm. If it is severed along with other parts, which have little importance in themselves, thus leaving less of the connection remaining, an extreme block, for example an 8:1 rhythm, may be established which remains constant or becomes more severe. Although cutting the left lateral and left ventrolateral portions separately does not result in complete dissociation, and although they are not capable of preserving complete coördination when either alone is left, yet the cutting of both invariably brings about complete and permanent A-V dissociation. Standstill of the ventricles follows the cutting of both of these parts in some cases; in others the A and V contract independently. The only other way this result can be produced in the alligator is to section the right lateral portion below the level of the A-V groove.

Influence of the vagus and sympathetic. Experiments were carried out to ascertain the relative influence of the right and left vagi and sympa-

thetics, as well as of the coronary nerve, on the normal heart and on those in which partial or complete block had been artificially produced. The results of stimulating the coronary nerve have already been discussed.

Gaskell (14) and Swett (10) have described the cardiac nerves in the alligator. In all experiments the four nerves were isolated and ligated. To investigate their influence on block, partial or complete block was produced by cutting the parts which, as above outlined, were found to be of importance for coördination.

Sympathetic stimulation. The sympathetic was stimulated just distal to its exit from the cardiac ganglion. Even the strongest currents failing to produce any effect, the heart was painted with a 0.5 per cent solution of atropin sulphate. Even after this, weak and moderate strengths of current had no effect on the rate or strength of the heart beat. With strong currents neither sympathetic produced any effect in six out of fourteen cases, while in the remainder an increase in amplitude and rate occurred only after long continued (30 to 45 seconds) stimulation. There was an increase in amplitude and acceleration of beat of about 10 per cent, which agrees approximately with the results obtained by Gault (15) on the turtle heart.

In certain cases when stimulation of the sympathetic resulted in accelerating the rate of the heart beat and stimulation of the coronary nerve in completely stopping it, the two were later stimulated simultaneously. The result was a clear antagonism between them, or an algebraic summation of their respective influences. The heart instead of being completely inhibited, as it was when the coronary nerve was stimulated alone, was reduced to a rate varying between 8 and 4 beats per minute.

Vagal stimulation. The usual effects on rate, strength and conductivity were obtained. As in other animals, both cold- and warm-blooded, the left vagus in many individual cases has little or no chronotropic influence but exerts a strong effect on the amplitude of contraction, together with a delayed A-V conduction. With appropriate strengths of current, stimulation of either vagus may produce partial block (dropped beats) although the influence of the left on conduction across the A-V connection is greater than the right.

Artificial block and cardiac nerve stimulation. As in other animals, the degree of block may be improved or made more severe by vagal action. We have several records of 4:3 and 3:2 rhythm being converted during stimulation to 1:1, which either persisted after the stimulation was stopped, or returned to the degree of block existing before the stimulation. The effect of the right vagus is definitely greater than that of the left in this regard, the left requiring a much stronger current in agreement with its average weaker chronotropic influence. These beneficial effects on block are of course due to the slower rate of impulse formation in the sinus node.

On the other hand we have records of cases of block in which the rate of the auricular contractions is not affected by vagal stimulation, but the degree of block is rendered much more severe. For example, a case of 5:1 rhythm was converted by stimulation of the left vagus with a moderate strength of current to 8:1 and 10:1 rhythm. This reverted to a 4:1 rhythm upon cessation of the stimulus and a little later to 5:1 as before the stimulation. No such effects could be produced by stimulation of the right vagus.

There was no differential effect demonstrable between the influence of the right and left accelerators upon block. Their influence on block was usually demonstrated only after long continued stimulation and often only after the stimulation was over. Depending upon the strength of current, the sympathetics either increase block (strong currents) by increasing the auricular rate, or decrease it (moderate strengths of current) owing to the positive dromotropic effect upon the A-V connection being greater than the chronotropic effect on the auricle. In some cases none of the cardiac nerves had any effect on ventricular contraction directly after complete block had been produced. Upon waiting thirty to forty-five minutes, however, stimulation of the sympathetic was found to have a beneficial influence, the ventricles being induced to beat again and in a coördinated fashion with the auricles, usually only so long as the stimulation lasted, although in certain cases the improvement persisted after the stimulation was stopped.

SUMMARY

1. The dorsal ligament (sino-ventricular connection) including the coronary nerve is not a conducting or coördinating mechanism. The stimulation of the proximal end of the coronary nerve, a branch of the right vagus, has no effect on the rhythm of the heart. Stimulation of the distal end has in many cases the same effect as stimulation of the vagus. The coronary nerve is a portion of the regulatory mechanism.

2. The dorsal and right dorsolateral portions of the A-V connection are the least important parts. Cutting either or both of them produces but slight and temporary incoördination. When they are the only parts connecting A and V coördinated A-V rhythm is lost.

3. The most important single part of the connection is the right lateral portion. When cut through at the level of the A-V groove the result is either partial block or a much lengthened A-V interval. When cut through in the upper third of the ventricle a complete block invariably results. The section of the right ventrolateral and of the left dorsolateral portions sometimes produces partial block.

4. Cutting through the main part of the ventral portion results in partial block. When the section includes the portion contiguous to the

left ventrolateral, complete block results. Cutting either the left lateral or the left ventrolateral portions singly results in permanent partial block. Sectioning both of them results in complete block.

5. The left lateral, the left ventrolateral and the right lateral are the most specialized portions of the connection. In them the function of conducting the impulse from A to V is most highly developed. This differentiation agrees with the structure of the connection.

6. Strong currents following administration of atropin are necessary to demonstrate any sympathetic influence. Both nerves are equally effective as accelerators, the left slightly more so than the right as an augmentor. The effect of simultaneous stimulation of the coronary and sympathetic nerves is an algebraic summation of their respective influences of inhibition and excitation.

7. Sympathetic stimulation with a moderate strength of current may improve partial block and may convert complete into partial block. With very strong currents, the degree of block is increased owing to the increased heart rate.

8. The right vagus has, on the average, a greater chronotropic effect than the left, while the influence of the left is usually greater than that of the right on force of beat and on A-V conduction. Vagal stimulation has little if any effect on complete block. Partial block may be increased, particularly following left vagal stimulation, by the direct inhibitory effect on conduction through the A-V connection; or it may be improved, particularly following right vagal stimulation, on account of the slower rhythm of impulse formation in the sinus node.

BIBLIOGRAPHY

(1a) LAURENS: Pflüger's Arch., 1913, cl, 139; (b) This Journal, 1916, xlii, 89.
(2a) LAURENS: Anat. Rec., 1913, vii, 273; (b) 1915, ix, 427.
(3) LAURENS: This Journal, 1917, xlii, 513.
(4) GASKELL: The involuntary nervous system. 1916.
(5) BARRY: Journ. Physiol., 1921, lv, 423.
(6) EYSTER AND MEEK: Physiol. Rev., 1921, i, 1.
(7a) HABERLANDT: Zeitschr. f. Biol., 1917, lxvii, 453; (b) 1922, lxxvi, 50.
(8) FREDERICQ: Arch. internat. de Physiol., 1913, xiii, 427.
(9) KÜLBS AND LANGE: Zeitschr. f. exper. Path. u. Therap., 1911, viii, 313; 1912, xi, 51
(10) SWETT: Anat. Rec., 1923, xxvi, 129.
(11) MACKENZIE: xviith Internat. Cong. Med., Sec. iii, Gen. Path. and Path. Anat., London, 1912, Sec. 121.
(12) VEIL: Journ. d. Physiol. et d. Pathol. gen., 1917, xvii, 196.
(13) HABERLANDT: Zeitschr. f. Biol., 1918, lxviii, 257.
(14) GASKELL AND GADOW: Journ. Physiol., 1884, v, 362.
(15) GAULT: This Journal, 1917, xliii, 22.

THE EXCRETION OF DYES AND OTHER SUBSTANCES IN THE FROG'S KIDNEY AND ITS BEARING UPON THE THEORIES OF RENAL SECRETION[1]

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Ever since Bowman (3) in 1842 stated that "it would indeed be difficult to conceive a disposition of parts more calculated to favor the escape of water from the blood than that of the Malphighian body," and since Ludwig, (12) advanced the view "that the urine formed by the glomerulus is a protein free filtrate of the blood plasma," the general view has held that most of the water and many of the other substances have been excreted through the glomeruli, and that much of the water has been reabsorbed through the tubules. On the other hand, it has been thought that many of the excretion products are excreted not in the glomeruli, but by the cells of the tubules themselves.

The latter view has been held particularly since Heidenhain (8) demonstrated by histological means that if sodium sulphindigotate is injected into an animal, the dye is found in the lumina of the tubules and, after death, within the cells of the tubules themselves.

This view seemed to be confirmed when Nussbaum (14) demonstrated that in the frog the glomeruli are supplied by blood from the renal arteries, while the tubules are chiefly supplied by blood from the renal portal vein. He found that if the renal arteries were ligated to stop glomerular excretion, sodium sulphindigotate still appeared in the urine although the amount of urine was scanty. Still further evidence for a secretion of

[1] The experiments embodied in this investigation represent part of a thesis submitted by R. N. Bieter in partial fulfilment of the requirements for the Degree of Master of Science at the University of Minnesota.

This paper was announced for reading before the Toronto meeting of the American Physiological Society, December 29, 1922, and was sent to the Editor of the AMERICAN JOURNAL OF PHYSIOLOGY at that time. As the writers learned in the meantime that some similar investigations had been carried on, but not published, by Prof. A. N. Richards, who had originated and published the method used in the investigation, the paper was withdrawn from the program of the Physiological Society and the JOURNAL in order to give Professor Richards opportunity to publish his results. The present paper was read before the American Physiological Society at the St. Louis meeting December 27, 1923.

dyes by the tubules was furnished by Rowntree and Geraghty (15), who perfused Ringer's solution containing phenolsulphonephthlein into the portal veins of large frogs, and found that the dye appeared in the urine.

The beautiful method which A. N. Richards (16) has introduced for the direct observation of the circulation and excretion in the frog's kidney, seemed to furnish an ideal method for the observation of these processes in the frog's kidney with especial reference to the light that it might throw upon the tests for kidney function now in use in clinical medicine.

The technique which we have employed is essentially the method of Richards. We have used a 500 watt nitrogen lamp as a source of light, and a liter flask filled with a very dilute copper sulphate solution containing about 5 grams of alum to condense and cool the light. In our later experiments we have eliminated entirely the use of knife and scissors and

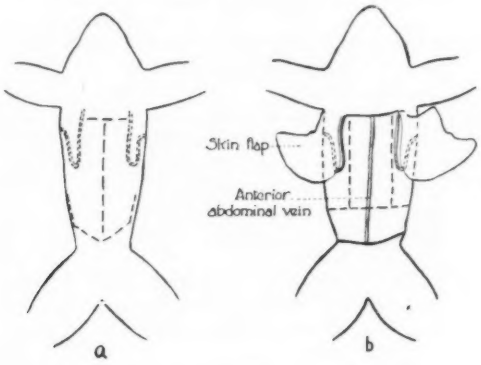

Fig. 1. *a*. Diagram of skin incision. - = Incision by cautery.
b. Diagram of abdominal muscle incision.

have completely controlled hemorrhage by making all skin incisions with a hot wire, and by avoiding the areas in which the vessels lie wherever this was possible.

We have confirmed Richards' observations that under normal conditions blood does not flow through all the glomeruli at once, and that the number of active glomeruli is markedly decreased by the subcutaneous injection of adrenalin, and increased by urea, theobromine, nitroglycerine, sodium nitrite and hypertonic sodium chloride. We were also able to show that sodium hippurate did not increase glomerular activity, but seemed even to depress it, while sodium benzoate definitely increased the number of active glomeruli and the rate of flow. This substantiates the views of H. B. Lewis (10) and of F. B. Kingsbury and W. W. Swanson (9), that sodium benzoate reaches the kidney as such and not in the form of the

hippurate; and it renders likely their hypothesis that the benzoate is synthesized into hippurate in the living kidney, the possibility of which had been shown by the experiments of Bunge and Schmiedeberg (4) in 1876.

TABLE 1

Showing effects of sodium benzoate and sodium hippurate on glomerular circulation

FROG NUMBER AND SEX	WEIGHT IN GRAMS	MILS INJECTED	EFFECT ON GLOMERULI IN MINUTES	EFFECT	DURATION IN MINUTES	2D INJECTION IN MILS	EFFECT
Sodium benzoate solution (1 mil = 100 mgm.)							
1 M	30	0.4	10	Increased flow in capillaries of glomeruli and tubules	30	0.5	Same
2 M	21	0.7	5	Increase in number of and speed in glomerular and tubular capillaries	3		
3 M	25	1.0	10	Increase in number of and speed in glomerular and tubular capillaries	30	0.5	Same
4 M	30	0.5	10	Increase in number of and speed in glomerular and tubular capillaries	20		
Sodium hippurate solution (1 mil = 140 mgm.)							
1 M	27	0.5	10	All glomerular circulation stopped; slight decrease in tubular circulation	60		
2 M	29	0.5	4	All glomerular circulation stopped; slight decrease in tubular circulation	25	0.5	Same
3 M	21	0.5	4	Glomerular circulation markedly decreased; tubular circulation decreased	20	0.5	All glomeruli stopped
4 M	25	0.5	5	Glomerular circulation decreased, stopped in many; tubular circulation decreased	30		

In our study of phenolsulphonephthalein we prepared a solution of the dye according to the method of Rowntree and Geraghty: to 0.6 gram of the powdered dye, 0.84 cc. of 2 N NaOH was added, and the total made up to 100 cc. with 0.75 per cent NaCl. This gave a 0.6 per cent solution. On another occasion we prepared the solution according to W. M. Clark (6), giving a 0.4 per cent solution. Three-tenths to six-tenths of a mil. of this were injected into the submaxillary lymph sac of the frog, taking care to clamp the site of injection for a few seconds after the removal of the needle, in order to prevent loss by leakage.

Within fifteen minutes increased flow through the glomeruli could be seen, and within a few minutes after this, the glomerular capsules could be seen to be distended with a light pink fluid, corresponding to an alkaline solution of phenolsulphonephthalein. In four animals whose circulation

TABLE 2

Showing the excretion of phenolsulphonephthalein

FROG'S NUMBER AND SEX	WEIGHT	MILS INJECTED	TIME FOR APPEARANCE		DURATION OF EXPERIMENT
			In glomerular capsule	In tubular lumen	
1 M	24	0.6	15 min.	30 min.	1 hr., 15 min.
2 M	27	0.4	20 min.	Not seen	30 min.
3 M	28	0.5	25 min.	40 min.	2 hrs.
4 M	30	0.5	15 min.	30 min.	1 hr., 30 min.
5 M	40	0.6	30 min.	40 min.	5 hrs., 30 min.
6 M	31	0.4	Not seen	40 min.	1 hr., 30 min.
7 M	25	0.3	10 min.	25 min.	2 hrs.
8 M	30	0.4	35 min.	50 min.	2 hrs.
9 M	43	0.6	15 min.	40 min.	4 hrs.

was already in a depressed condition before injection no change was noted; and in only one of these was the dye visible in the glomerular capsules.[2]

In all the animals the dye appeared in the tubules five to forty minutes later than in the glomerular capsules, and always of a much deeper hue. (Fig. 3.)

In studying the excretion of sodium sulphindigotate we made up a 1 per cent solution and injected 0.3 to 0.6 of a mil. into the submaxillary

[2] A preliminary report of the excretion of phenolsulphonephthalein and of sodium sulphindigotate in the frog's glomeruli was made by A. D. Hirschfelder and R. N. Bieter, Proc. Soc. Exper. Biol. and Med., N. Y., 1922, xix, 351 and 415. The results of similar experiments which were made independently about the time of our own observations were reported by A. N. Richards and Wearn at the May, 1923, meeting of the American Society for Clinical Investigation, and were published in the Proceedings of this Society (Journ. Amer. Med. Assoc., 1923, lxxx, 1644). Richards and Wearn further confirmed their findings by tapping the glomerular capsule with a capillary tube and demonstrating the color in the glomerular fluid thus removed.

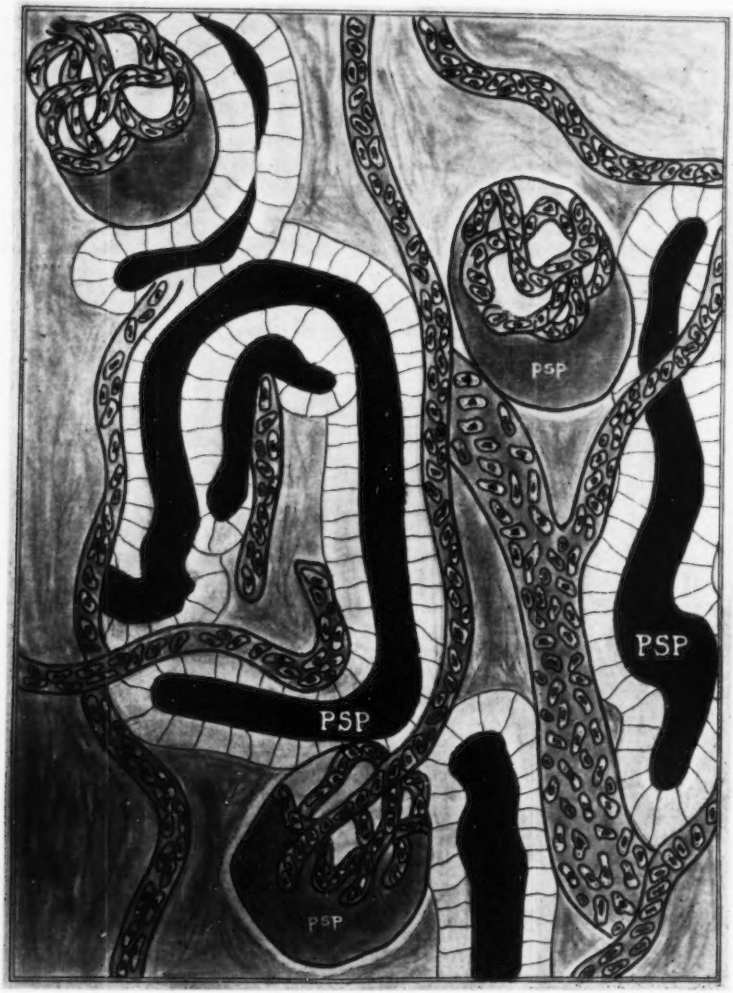

Fig. 2. Drawing from an area of frog's kidney showing the excretion of phenolsulphonephthalein.

The relative intensities of the dye in the tubules are deeper than in the glomerular capsule.

PSP. = Phenolsulphonephthalein in the lumen of tubules.

psp. = Phenolsulphonephthalein in glomerular capsule.

lymph sac. The glomerular capsule began to bulge out and took on a blue color in from fifteen to thirty minutes. But in one case with depressed circulation the dye was not seen in the capsule for one hour and thirty-five minutes, and from twenty to sixty minutes later it could be seen in the lumina of the tubules, but with a much deeper hue (fig. 2). In all our observations it seemed clearly confined to the lumina of the tubules and we could not see evidence that it entered the cells or stained their nuclei as shown in the figures of Heidenhain and many subsequent authors. It is not improbable that their pictures were post-mortem phenomena.

That these dyes are excreted by the glomeruli alone and not by the tubules is shown by the following experiments:

Branches of the right renal artery going to the lower pole of the right kidney are exposed by drawing the left kidney aside and up over the

TABLE 3

Showing the excretion of sodium sulphindigotate

Injections of sodium sulphindigotate, 1 per cent solution

FROG'S NUMBER AND SEX	WEIGHT	MILS INJECTED	TIME FOR APPEARANCE		DURATION OF EXPERIMENT
			In glomerular capsule	In tubular lumen	
1 M	30	0.4	30 min.	1 hr.	3 hrs.
2 M	20	0.5	15 min.	30 min.	2 hrs.
3 M	21	0.5	20 min.	45 min.	1 hr., 15 min.
4 M	22	0.5	20 min.	30 min.	1 hr.
5 M	20	0.3	20 min.	30 min.	1 hr., 45 min.
6 M	30	0.4	1 hr., 35 min.	Not seen	2 hrs., 15 min.
		(Circulation depressed from beginning)			
7 M	25	0.4	15 min.	35 min.	2 hrs.

right kidney without touching the right. They are then seared across by touching each of them with the tip of a fine heated iron wire. When the kidney is now placed under the microscope in the usual manner it can be found that the circulation through the glomeruli, which are situated in the lower part of the kidney, has entirely ceased, and the red blood cells remain perfectly still in the glomerular capillaries. On the other hand, there is a rapid circulation through the veins to the renal tubules, which are supplied from the renal portal vein. In the upper part of the kidney whose arterial supply is still intact, the blood is flowing through the glomerular capillaries in the usual manner.

If phenolsulphonephthalein or sodium sulphindigotate is now injected into the submaxillary lymph sac, the dye is found after the usual interval in the capsules of the active glomeruli (Fig. 3), and in the tubules in the portion of the kidney whose arterial blood supply is intact; but the lower

part of the kidney whose arterial supply has been cut off and whose glomeruli are stagnant remains entirely free from dye.

This is in striking contrast to the experiments of Nussbaum (14) with sodium sulphindigotate, since he found that frogs whose renal arteries were ligated still excreted the dye,—a fact which he interpreted as evidence that it was excreted through the tubules. It is also in contrast to the experiments of Rowntree and Geraghty who perfused phenolsulphonephthalein containing solutions through the renal portal veins of large

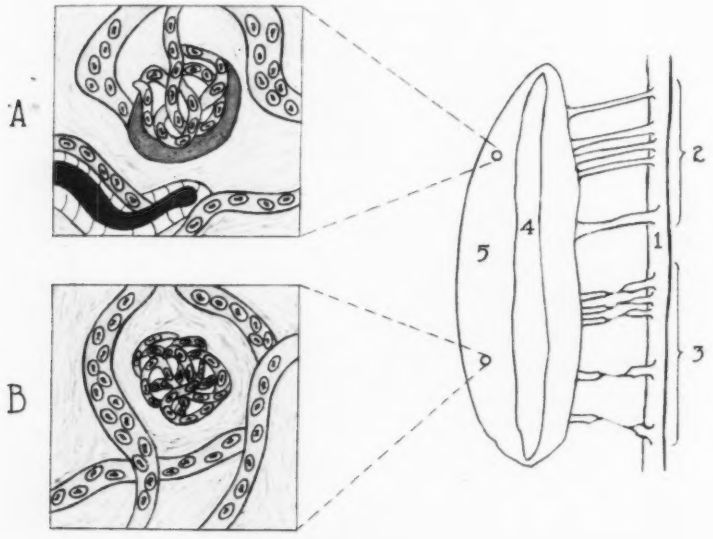

Fig. 3. Showing cauterization of renal arteries.

A, active glomerulus and tubule from upper pole; *B*, inactive glomerulus from lower pole; *1*, abdominal aorta; *2*, intact renal arteries; *3*, cauterized renal arteries; *4*, adrenal gland; *5*, kidney (right).

frogs and found the drug in the urine, although subsequent injection of Prussian blue showed that none of the fluid injected from the renal portal had entered the tubules.

The results of some of our experiments throw light on these discrepancies. In some of our frogs, in which branches of the renal arteries were cauterized, the glomeruli in the corresponding areas remained stagnant and the areas remained free from color for an hour or so. After a lapse of this time, however, an active circulation again became visible in the

TABLE 4

Showing the effects of cautery of renal arteries on the excretion of dyes

FROG'S NUMBER SEX AND WEIGHT	CAUTERY OF RENAL ARTERIES	RESULTING PICTURE	AMOUNT OF DYE AND TIME	APPEARANCE IN CAPSULE	APPEARANCE IN TUBULE	ONE HOUR LATER		TWO HOURS LATER	
						Glomer.	Tubule	Glomer.	Tubule
Phenolsulphonephthalein									
1 M 25	Lower $\frac{2}{3}$	Upper $\frac{1}{3}$ Lower $\frac{2}{3}$	0.5 at –11:00	*11 : 20* None	*12 : 00* None	+ –	++ –	+ –	+ –
2 M 32	Lower $\frac{2}{3}$	Upper $\frac{1}{3}$ Lower $\frac{2}{3}$	0.7 at 11:30	*11 : 50* None	*12 : 20* None	+ +	+ +*	+ +	+ +
3 M 26	Lower $\frac{1}{2}$	Upper $\frac{1}{2}$ Lower $\frac{1}{2}$	0.6 at – 9:50	*10 : 40* None	*10 : 20* None	++ –	+ –	+ –	+ –†
4 M 27	Lower $\frac{2}{3}$	Upper $\frac{1}{3}$ Lower $\frac{2}{3}$	0.5 at –11:15	*11 : 25* None	*11 : 55* None	+ –	+ –	+ –	+ –
5 M 27	All branches	No glom. flow	0.7 at 10:00	None	None	None	None	None	None
6 M 23	Lower $\frac{1}{2}$	Upper $\frac{1}{2}$ Lower $\frac{1}{2}$	0.5 at –10:10	*10 : 25* None	*10:30* None	+ –	++ –	+ –	+ –
7 M 30	Lower $\frac{2}{3}$	Upper $\frac{1}{3}$ Lower $\frac{2}{3}$	0.7 at –11:00	*Not seen* None	*11 : 15* None	+ –	++ –	+ –	+ –†
8 M 23	Lower $\frac{1}{2}$	Upper $\frac{1}{2}$ Lower $\frac{1}{2}$	0.5 at –11:30	*Not seen* None	*12 : 00* None	+ –	++ –	+ +	+ +*
9 M 25	Lower $\frac{1}{2}$	Upper $\frac{1}{2}$ Lower $\frac{1}{2}$	0.5 at –12:00	*12:15* None	*12 : 40* None	+ –	+ –	+ –	+ –
10 M 23	All branches	No glom. flow	0.5 at 12:30	None	None	None	None	None	None
Sodium sulphindigotate									
1 M 24	Lower $\frac{2}{3}$	Upper $\frac{1}{3}$ Lower $\frac{2}{3}$	0.5 at 10:45	*11 : 15* None	*11 : 25* None	+ None	++ None	+ None	++ none
2 M 24	All branches	No glom. flow	0.5 at 10:15	None	None	None	None	None	None

TABLE 4—*Concluded*

FROG'S NUMBER SEX AND WEIGHT	CAUTERY OF RENAL ARTERY	RESULTING PICTURE	AMOUNT OF DYE AND TIME	APPEARANCE IN CAPSULE	APPEARANCE IN TUBULE	ONE HOUR LATER		TWO HOURS LATER	
						Glomer.	Tubule	Glomer.	Tubule
Sodium sulphindigotate—*Concluded*									
3 M 27	Lower $\frac{2}{3}$	Upper $\frac{1}{3}$	0.5 at	*10:05*	*10:10*	+	++	+	+
		Lower $\frac{2}{3}$	9:50	None	None	+	+*	−	−
4 M 25	Lower $\frac{1}{2}$	Upper $\frac{1}{2}$	0.5 at	*9:25*	*9:30*	+	++	+	+
		Lower $\frac{1}{2}$	9:05	None	None	+	+	+	+*
5 M 28	Lower $\frac{1}{2}$	Upper $\frac{1}{2}$	0.5 at	*10:15*	*10:25*	+	++	+	+
		Lower $\frac{1}{2}$	10:00	None	None	−	−	−	−
6 M 30	All branches	No glom. flow	0.6 at 12:00	None	None	None	None	None	None
7 M 27	Lower $\frac{1}{2}$	Upper $\frac{1}{2}$	0.5 at	*1:25*	*1:35*	+	++	+	+
		Lower $\frac{1}{2}$	1:00	None	None	+	+*	−	−

+ = glomerular activity.
− = no glomerular activity.
+ = appearance of dye in glomeruli or tubule.
− = absence of dye.

* Glomerular activity began as indicated in the ligated areas, lasting for a variable length of time. The dye appeared likewise in the tubules in these areas, during the time of glomerular activity.

† Circulation depressed from beginning.

capillaries of some of the glomeruli, and after the usual interval the dye appeared in their capsules and in the lumina of the tubules in the corresponding area, but nowhere else in the ligated areas. It is evident that this was due to the establishment of a collateral circulation; and it is probable that in Nussbaum's experiments which were extended over several days, a similar collateral circulation was established. In fact, Adami (1) found, in attempting to confirm Nussbaum's work, that after ligature of all the visible renal arteries, indigo carmine and powdered vermillion could still be carried by the circulation into the capillary loops of the glomeruli. He found this especially true for the mid-portion of the kidney and explains Nussbaum's negative results in this regard on the probability that Nussbaum may have examined only sections from the upper or lower poles of the kidneys.

These same findings of Adami's may explain the results of Rowntree and Geraghty also, although they may be accounted for on a different

basis as well. In their experiments there was ample opportunity for absorption of some of the phthalein containing solution by the lymph channels, or by a slowly established and slight backward ooze into the glomeruli through the efferent vessels. This might have been too slow and slight to have shown with their subsequent injection of Prussian blue, for after injecting the latter to determine whether the injection fluid penetrated the glomeruli, they removed the kidney at once and fixed it in alcohol. Bainbridge has shown that the anastomotic circulation backward into the glomeruli through the efferent branches proceeds very slowly.

Our own results above mentioned seem to exclude entirely the excretion of these dyes through the tubules.

As regards the question of the absorption of water from the tubules, considerable light is thrown upon the question by the depth of color which can be seen in the kidneys. If we consider the fact that the diameter of the glomerular capsule is about four times that of the lumen of the tubules and that in spite of this, the color of the dye in the tubule is three or four times that in the capsule, it would appear that the concentration of the dye in the tubule must be at least twelve to sixteen times that in the glomerulus.

The color of the dye is very deep in the convoluted tubules, and seems to deepen progressively as one passes from the proximal to the distal loops. This would indicate that concentration begins in the proximal convoluted tubules.

Insofar as we know, these experiments furnish the first absolutely objective proof that has been advanced for the prevalent theory that the urine is excreted in dilute form through the glomeruli and is concentrated in the tubules by reabsorption of water.

Our results are in partial accord with the ingenious experiments recently published by G. A. Clark (5) who studied the excretion of sugar upon the perfused frog's kidney. He found that the glomeruli when perfused through the arterial circulation were absolutely permeable to sugar, but that some sugar was reabsorbed by the tubules. On the other hand, in a number of experiments he found that the urine contained exactly the same concentration of sugar as the fluid perfusing the glomeruli; and he concludes from this that no absorption of water occurs in the tubules. However, since he has shown that the tubules themselves are capable of reabsorbing glucose, he has not excluded the possibility that they had reabsorbed both water and glucose in the same ratio; and, on the other hand, since the very experiments of his series which he quotes in this connection give figures similar to whose which he mentions in other experiments in which the tubules had been poisoned by the injection of mercuric salts into the portal vein, it is also possible that in these experiments the tubules were not functioning at all.

In our own experiments the evidence seems incontrovertible that the tubules were functioning and were reabsorbing the water, but not secreting or reabsorbing the dye.[3]

SUMMARY AND CONCLUSIONS

1. Observation of the frog's kidney by Richards' method shows that phenolsulphonephthalein and sodium sulphindigotate are excreted through the frogs' glomeruli and can be seen in the capsular fluid.

2. They do not appear in the glomeruli or the tubules when the circulation through the glomeruli has been cut off, though the tubular blood supply is intact, and hence, they are not excreted by the tubules.

3. Insofar as can be concluded from studies upon the frog's kidney, the phenolsulphonephthalein test of renal function is a test of pure glomerular function and not of tubular function.

4. The dyes are markedly concentrated within the lumina of the tubules.

5. These experiments furnish the first absolutely objective proof that has as yet been advanced for the theory that the urine is excreted in dilute form in the glomeruli and concentrated in the tubules by the reabsorption of water which occurs at least in the descending loops of Henle.

BIBLIOGRAPHY

(1) Adami: Journ. Physiol., 1885, vi, 382.
(2) Bainbridge, Menzies and Collins: Journ. Physiol., 1914, xlviii, 233.
(3) Bowman: Phil. Trans. Roy. Soc., London, 1842, 74.
(4) Bunge and Schmiedeberg: Arch. f. Exper. Path. u. Pharm., 1876 vi, 233.
(5) Clark: Journ. Physiol., 1922, lvi, 201.
(6) Clark: The determination of hydrogen ions. Baltimore, 1920.
(7) Cushny: For complete bibliography. The secretion of urine. London and New York, 1917.
(8) Heidenhain: Arch. f. d. gesammt. Physiol., 1874, ix, 1. Hermann's Physiologie, 1883, v, 279; Arch. f. mik, Anat., 1874, x, 1.
(9) Kingsbury and Swanson: Arch. Int. Med., 1921, xxviii, 220.
(10) Lewis: Journ. Biol. Chem., 1914, xviii, 225.
(11) Lewis and Karr: Ibid., 1916, xxv, 13.

[3] Since our experiments were completed our attention has been called to an article by Woodland (Journ. and Proc. Asiatic Soc., 1922, N. S. xviii., no. 2, 85) in which it is claimed that there is very little anastomosis between the renal arterial capillaries and the renal portal vein capillaries; and that it is the circulation through the arterial circulation, only, which is responsible for the entire renal function. He also believes that the glomerulus is an automatic mechanism for regulating the kidney blood supply and thus preventing an excessive action on the part of the tubules.

Our own experiments as well as those of Richards and many of the older observations prove conclusively that the glomeruli excrete the dyes in dilute solution and we have shown here that the urine is concentrated in the tubules; and these observations are not in the least affected by Woodland's contentions.

(12) Ludwig: Wagner's Handworterb. d. Physiol., 1844, ii, 637.
(13) V. Mollendorff: Asher and Spiro's Ergebn. d. Physiol., 1920, xviii, 246.
(14) Nussbaum: Arch. f. d. gesammt. Physiol., 1878, xvi, 139; ibid., 1878, xvii, 580.
(15) Rowntree and Geraghty: Journ. Pharm. Exper. Therap., 1910, i, 579; Arch. Int. Med., 1912, ix, 284.
(16) Richards: Amer. Journ. Med. Sci., 1922, clxiii, 1.

(For preliminary reports see: Hirschfelder and Bieter: Proc. Soc. Exper. Biol. and Med., 1922, xix, 351, 415.)

AN APPARATUS FOR ELICITING AND RECORDING THE PATELLAR TENDON REFLEX

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It is evident that if one is to study the patellar tendon reflex quantitatively it should be elicited by a stimulus of uniform intensity at regular intervals. Since this reflex is subject to variations due to very slight environmental changes it is desirable to have an apparatus which is capable of delivering stimuli automatically. With these ideas in view the apparatus described in this paper has been developed and thoroughly tested. The results obtained are so satisfactory that it seems desirable to give a rather detailed description of it.

The apparatus consists of four parts as follows: 1, the power unit; 2, the hammer unit; 3, the chair unit; 4, the recording unit.

The accompanying photograph shows the units assembled as they are used in carrying out the various experiments which have been performed.

The power unit. The first point to be considered in constructing the power unit is the frequency with which the stimuli are to be delivered. Before deciding upon an interval between stimuli best suited to the elicitation of the reflex, the time was observed which was necessary for the reflex to be completed. It was found that from 8 to 10 seconds were adequate for the completion of the reflex, allowing at the same time sufficient rest to obviate fatigue as a significant factor. Figure 2 is a working drawing of the power unit.

The desired frequency is secured by means of a system of pulleys together with resistance in the motor circuit. A 1750 R.P.M. constant speed motor, *A*, equipped with a steel pulley, *B*, is belted to a wood pulley, *C;* the shaft of the latter is fitted with a steel pulley, *D*, which is connected to a system of belt-driven reducing pulleys, *E*, *F*, *G*. In order to increase the range of speeds which can be obtained a parallel bank of lamps, *H*, is placed in series with the motor. (If desirable, a sliding resistance may be substituted for the lamp bank.) By varying the number of lamps in circuit the interval between stimuli can be made to vary from 4 to 12 seconds.

In order to change the circular to longitudinal motion an eccentric, *I*, is mounted upon the last pulley. An eccentric rod, *J*, is attached to a

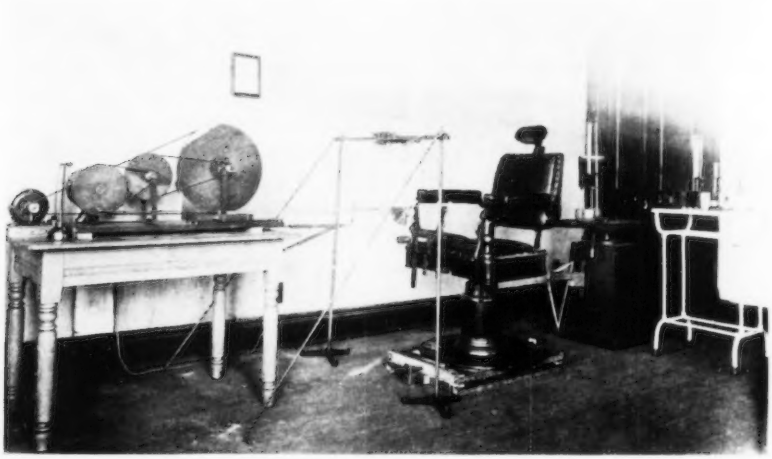

Fig. 1. An apparatus for eliciting and recording the patellar tendon reflex.

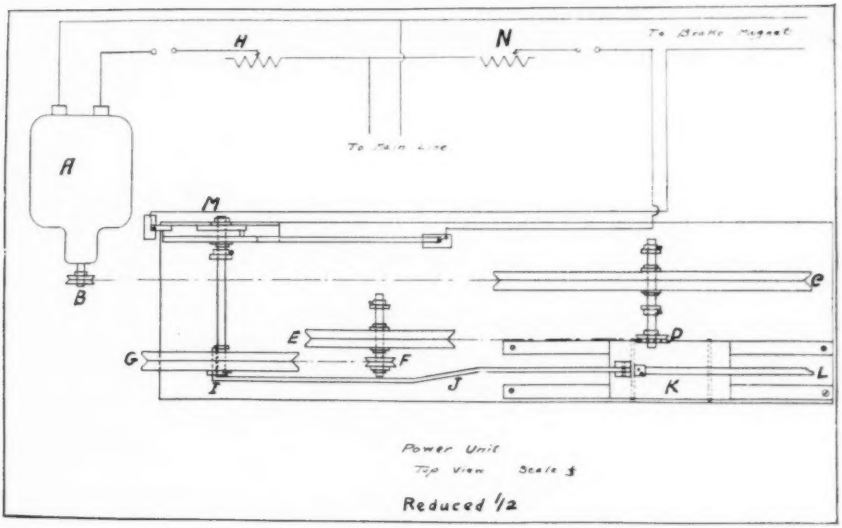

Fig. 2

carriage, *K*, which slides on a double track. It is held in place by a support at each end, running under the track.

A wood bar, *L*, is hinged to the upper surface of the carriage well toward the end to which the eccentric rod is attached. Upon the distal end of this bar a hook is attached for engaging a tripping device upon the hammer handle. The engaging bar is firmly supported by a deep grooved guide at the extremity of an iron bracket fastened to the end of a table. The power unit complete is mounted upon a small table.

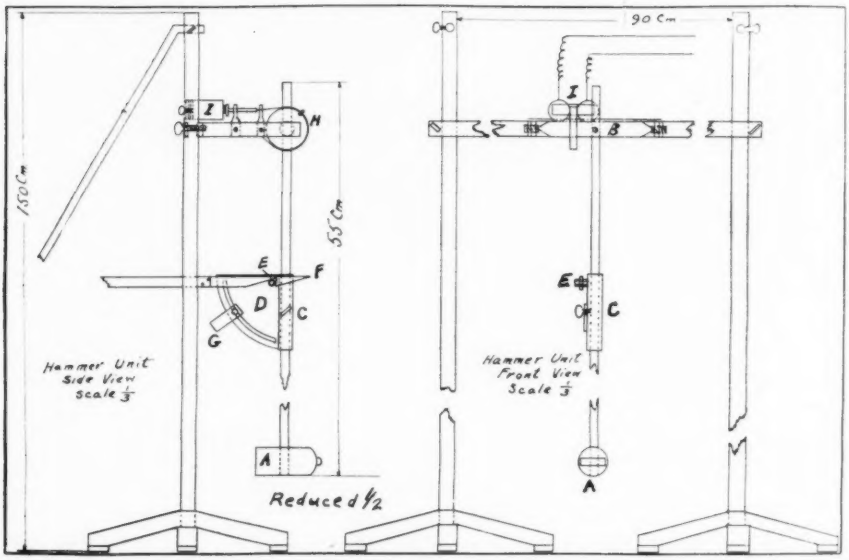

Fig. 3

The hammer unit. In constructing the hammer unit the principal points to be considered are adjustability and adequacy of striking force.

A steel hammer, *A*, weighing 824 grams is suspended upon a handle from an axle, *B*. The hammer handle passes through an axle, being securely held in place by a thumb screw. This arrangement makes the length of the hammer handle adjustable. The axle swings in a bracket provided with two brass bearings which are adjusted so as to offer as little resistance as possible.

In order that the hammer may swing automatically an engaging and tripping device is attached to the hammer handle. This device consists of a flattened metal sleeve, *C*, which is held in place upon the hammer handle by a thumb screw. To one side of the sleeve a brass quadrant, *D*, is fastened which presents a flat side parallel to the engaging hook.

Near the central angle of the quadrant a lug, *E*, with a freely moving brass sleeve is bolted perpendicular to the quadrant. This serves as an attachment for the engaging hook, *F*. A slot is cut in the quadrant near its circumference and parallel to it. Opposite the engaging hook a piece of metal the width of the hook, but somewhat shorter, is fastened to the arm. This serves as a guide so that when the hook engages the lug the quadrant passes between the guide on the one side and the hook on the other. A thumb screw passing through the sleeve to which the quadrant is fastened holds the engaging and tripping device firmly in position on the hammer handle. A metal strip, *G*, attached to the quadrant by means of a thumb screw serves as the tripping device. When the lug is engaged and the hammer pulled back the metal trip comes in contact with the inferior side of the hook arm, raising, and thus disengaging it, as the hammer continues to move toward the power unit. The hammer is then free to fall.

The power unit is placed at such a distance from the hammer unit that when the wood carriage reaches the distal extent of its excursion the hook on the hook arm drops over the lug on the quadrant. As it approaches the proximal end of its excursion the hammer is released and is free to fall against the patellar tendon.

It was found that when the hammer struck the tendon it rebounded, thus delivering a series of stimuli of diminishing force. This is objectionable since it not only serves as a source of annoyance but in case the subject is hyperirritable the first or second rebound may elicit the reflex. These secondary stimuli might obviously serve to augment or depress the desired reaction. In order to prevent this rebound an electrically controlled brake is mounted on the axle from which the hammer swings. A fiber drum, *H*, is forced tightly on the axle near its center; around the drum a band spring is placed. One end of the spring is securely fastened to a brace while the other end is attached to a soft iron bar which serves as an armature. The spring covers about two-thirds of the drum. Two electro-magnets, *I*, mounted upon a cross-bar behind the hammer draw the armature forward, thus tightening the spring and preventing a rebound of the hammer. The distance from the magnets to the armature is adjustable. In practice the adjustability of the magnets was found to be important.

The brake is needed an instant after the hammer strikes. This is determined by a timing device (*M*, fig. 2) attached to the axle of the eccentric pulley. The timing device consists of a wood pulley encircled by two brass strips, one of which completely encircles the pulley and through its connection with a copper brush serves as a constantly engaged terminal. The brush of the other terminal slides upon a second brass band partially encircling the pulley. The two bands are connected by

a metallic bridge. The gap in the second band is so placed as to give a break of the desired length and at the desired time, while the armature is passing over the dry wood of the pulley. The current is kept interrupted through most of the circuit by way of avoiding heating of the brake magnets. One-hundred-ten volts are used in all circuits. The strength of the magnets is controlled by a parallel bank of lamps (*N*, fig. 2) in series with the timer. A sliding resistance might be substituted for the lamp bank. The entire mechanism is suspended from a cross-bar firmly fastened to two uprights by thumb screws.

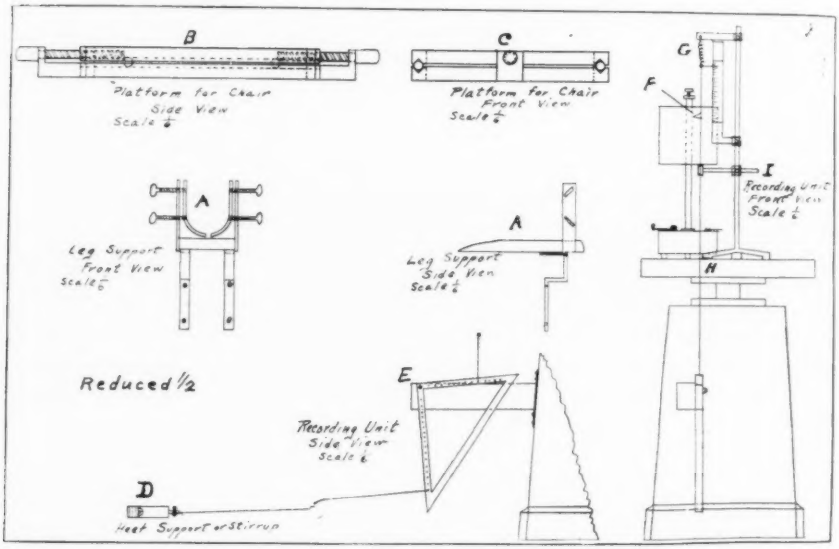

Fig. 4

The hammer unit includes three adjustments for varying the intensity of the blow: a, changing the length of the hammer handle; b, raising or lowering the engaging quadrant; c, adjusting the trip in the slot. The adjustability of the cross-bar from which the hammer swings makes it possible to compensate so that the height of the engaging arm remains constant.

The chair unit. The subject is seated in a barber's chair from which the foot-rest was removed. The necessary tension for the patellar tendon is secured by elevating the thigh somewhat above the horizontal and holding the leg slightly back of the vertical by its attachment to a spring that forms part of the recording device. The lower end of the thigh rests in a comfortable holder, at the distal end of which is a U-shaped

guide (*A*, fig. 4) so constructed that both the width of the U and the angles of its sides can be adjusted. Although this permits more or less forced lateral displacement of the leg, it is found in actual practice quite adequate to keep the point upon which the hammer impinges in a constant position.

The chair unaltered furnishes up, down and lateral adjustments. To permit forward and backward adjustments the chair unit is mounted upon a double base (*B* and *C*, fig. 4), between which are four steel balls running in an iron groove. Wood screws in front and behind maintain the adjustment.

The recording unit. A stirrup, *D*, is attached by a set screw to the heel of the subject's shoe. From the stirrup runs a wire to the vertical arm of a triangular device, *E*, suspended at its right angle. To the horizontal arm of the triangle a second piece of piano wire is attached which runs to a stylus, *F*, suspended from a wire spring, *G*. The triangle pro-

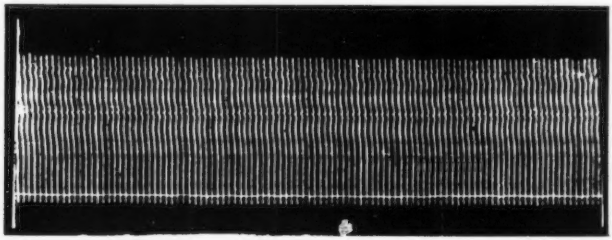

Fig. 5. A record showing the constancy of the intensity of the stroke and the regularity with which the hammer falls.

vides a means of changing the relation of the excursion of the heel to the excursion of the stylus. In practice it has been found desirable to work with relatively low kicks to permit registering the marked augmentations frequently encountered.

The stylus spring plays an important rôle in bringing the heel back after each excursion exactly to its original position. By changing the tension on the spring the resistance offered to the reflex may be varied.

The wire from the horizontal arm of the triangle passes through a screw eye at *H* (not shown in drawing) and under a pulley, *I*. The pulley serves as a guide so that the stylus writes perpendicularly on the drum. A stop is clamped on the wire just below the screw eye to prevent fling. This device serves also to keep the initial tension constant.

Time markers and signal magnets may be added as occasion demands. The entire unit is mounted on a heavy pedestal.

Constancy of intervals and force of strokes. The constancy of the intensity and the regularity of the strokes are illustrated by figure 5. This record was made by allowing the hammer to fall against a lever hinged to the floor to take the place of the leg of a subject. Resistance was supplied by a rubber band.

The records read from right to left due to the fact that the base line is at the top of the record. This method was adopted to avoid the use of a second pulley.

Experience with this apparatus has shown that it is capable of eliciting the patellar tendon reflex to a nicety and of accurately recording any changes which are exhibited by it. Its complete automaticity, permitting the elimination of certain extraneous environmental variables is a valuable feature. Its sturdiness of construction and delicacy of performance render it a valuable addition to class room armamentarium both in physiology and pharmacology.

It is a pleasure to express my appreciation for various suggestions received from Professors A. P. Weiss and R. G. Hoskins. I also wish to thank Mr. George Woodward and Mr. Carl Effler for their suggestions and assistance throughout the construction of this apparatus.

THE EFFECT OF SLEEP UPON THE PATELLAR TENDON REFLEX

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A number of observers have noticed that during sleep the patellar tendon reflex is either greatly diminished or absent altogether. Lombard (1) in 1887 found this to be true and more recently Lee and Kleitman (2) have made similar observations.

During the course of a number of experiments upon the patellar tendon reflex, three subjects fell asleep. The records obtained from these subjects are so striking graphically as to seem worthy of report.

In this experiment by means of an apparatus described in a preceding report (3) uniform stimuli were delivered to the patellar tendon automatically at a constant rate of eight per minute. Lee and Kleitman, in reporting some recent work in which the knee jerk was used as a criterion of neuromuscular tonus, stated that since the whole leg is used as a lever the method is necessarily crude. As a matter of fact, several months' experience has shown that the chief difficulty lies in the sensitiveness of the method. Although the leg does offer considerable inertia it is moved by a correspondingly large muscle mass. In case of an untrained subject the succession of knee kicks varies in a surprising way. Every stimulus which evokes emotion or interest, however small, is reflected in the graphic record. In using the apparatus one comes quickly to the realization that the spinal cord of a waking subject is never in a condition of rest or of steady activity but is subject to an ever changing ebb and flow of impluses. Even in case of the trained subject whose graph is shown in figure 1 considerable variability is evident although all the mechanical conditions were kept constant.

Figure 1 is the tracing made by a subject during two sleeping and three waking periods. He is a man of 32 years, in good health. He had, however, been undergoing insomnia for several nights. He is well trained as a subject in knee-kick experiments, hence the readiness with which he fell asleep after being awakened. Lee and Kleitman (2) have shown that this reflex as such is not significantly affected by preceding insomnia.

The first waking period, *A*, lasted for eight minutes, after which the subject fell asleep for thirty-five minutes, *B*. The subject was observed

to readjust his posture somewhat at various times but not sufficiently to throw the patellar ligament out of adjustment in relation to the stimulating hammer. During these postural readjustments at the beginning of the first sleep periods low kicks were registered. At intervals through the thirty-five minutes of sleep the subject readjusted himself somewhat at various times, each time registering one or more low kicks. The subject awoke the first time of his own accord. Just previous to the waking period, *C*, the subject was observed to readjust his posture, at the same time delivering very low kicks. This period of readjustment was the first indication that the subject was awakening. As soon as he was completely

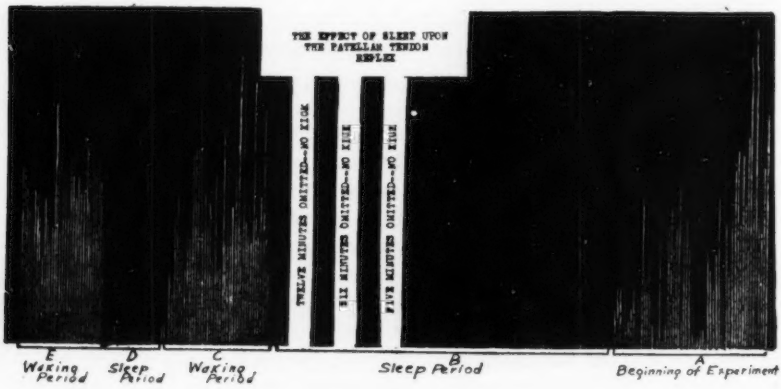
THE EFFECT OF SLEEP UPON THE PATELLAR TENDON REFLEX
TWELVE MINUTES OMITTED--NO KICK
SIX MINUTES OMITTED--NO KICK
SEVEN MINUTES OMITTED--NO KICK
E Waking Period
D Sleep Period
C Waking Period
B Sleep Period
A Beginning of Experiment

Fig. 1

awake he engaged in conversation, delivering at this time kicks very similar to those given during the previous waking period. After six minutes the subject again fell asleep and remained thus for four minutes, *D*. This last sleep period was terminated by the operator who spoke to the subject. Wakefulness followed at once, accompanied by kicks very similar to those delivered during the previous waking periods. The subject continued to deliver normal kicks for four minutes, at which time the experiment ended.

Figure 2 shows four curves made by the three subjects used in this experiment. The curves show the condition of the reflex previous to, during and after sleep. In order to render more intelligible the general trend of individual curves, use was made of the well known smoothing device. For most purposes plotting the average of each succeeding eight kicks is sufficient to submerge the effect of transient factors. This device has been utilized in plotting the curves shown in figure 2.

The chief points of interest brought out in this study were the following: It was observed that as a subject fell asleep the patellar tendon reflex

gradually decreased, finally disappearing. Whenever the subject was sufficiently awake to readjust his posture the reflex returned, the degree of return depending upon the degree of wakefulness. When the sleep

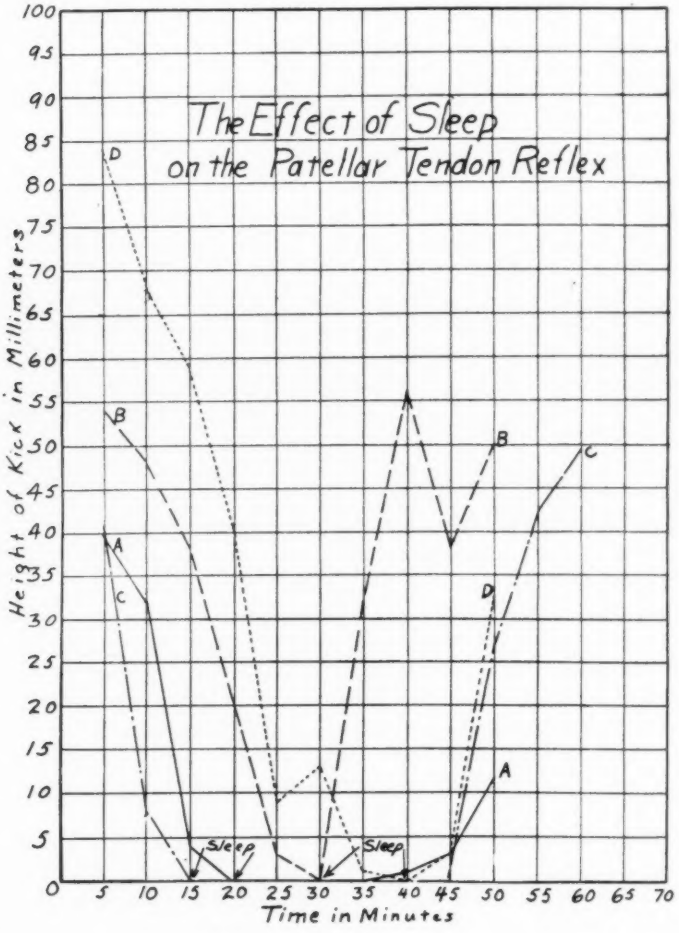

Fig. 2

was interfered with by some external cause the reflex returned at once. When the awakening was spontaneous the reflex gradually returned to normal. There appears to be some degree of correlation between the reflex and the depth of sleep.

I wish to thank Prof. R. G. Hoskins for various suggestions throughout these experiments. I also wish to thank Mr. George Woodward for his assistance during the experiments.

BIBLIOGRAPHY

(1) LOMBARD: Amer. Journ. Psychol., 1887, i, 5.
(2) LEE AND KLEITMAN: This Journal, 1923, lxvii, 141.
(3) TUTTLE: This Journal, 1924, lxviii, 338.

A NOTE ON THE DETERMINATION OF CHLORIDE-IONS

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The object of this paper is to describe some experiments performed in the spring of 1921, which have remained unpublished on account of illness of the writer; since then analytical methods based on the same principle, the concentration chain, have been published by Neuhausen and Marshall (1); it will be seen that the work reported in this paper differs in its object and technique from the experiments made by these investigators; my plan was to develop a method which would make it possible to register continuously changes in the chlorine-ion concentration of body fluids and living tissue in situ.

A chain of the type

Ag/ Ag Cl/ Chloride c_1/ bridge/ Chloride c_2/ Ag Cl/ Ag

has been described by Afanasjew (2), Biron (3), Bronsted (4), Dunin-Borkowski (5), Galeotti (6), Jahn (7) and Sackur (8). The half-cell Ag/ Ag Cl/ n K Cl—has a constant e.m.f. of 0.513 volt + 0.002(273–t°) and has been studied by Goodwin (9), Jahn (7) and Küster and Thiel (10). It is a chain of the second order based on this principle: the e.m.f. of a half-cell containing a metal and a (practically) insoluble metal salt and a salt in solution with the same anion as the insoluble metal salt, depends entirely on the concentration of the anion in solution; the concentration of the other ions being constant (11). The calculation for such a chain is the same as for a chain of the first order (12):

$$\text{e.m.f.} = \frac{2\,u}{u\,v}\,R\,T\ln\frac{C_1}{C_2}$$ where C_1 and C_2 are the anion-concentration;

the calculated value is very near the experimental value, i.e., within 1.5 millivolts as shown by Nernst (12). For all practical purposes in physiologic work a calculation of the chlorine-ion concentration from the observed e.m.f. is not necessary; it is sufficient to standardize the silver chloride electrode against a chloride solution of known dissociation and concentration; the observed e.m.f. is then plotted against the known molar Cl concentration. The value of the chlorine-ion concentration in future ex-

periments is easily extrapolated from this curve. Such curves were drawn during this work. Merck's blue label reagent K Cl was recrystallized three times and the solutions used made by dilution from one standard 0.1 n solution. Baker's silver in the form of wire and thin sheets was covered electrolytrically with Ag, the current density varying with the surface of the electrode yet always great enough to deposit 0.01 mm. Ag in three hours; the electrode was carefully cleaned by rinsing and immersing in distilled water. To coat the electrodes with Ag Cl a 25 per cent solution of hydrochloric acid (Baker's, redistilled) and a current of 3-5 milli-ampere were found to be the best for the small electrodes. The whole procedure, including the standardization as well as subsequent experiments, was performed by artificial light; however, it was found that the presence of a reddish brown color probably due to silver sub-chloride does not effect the potential. The finished and washed electrodes were kept in closed vessels each containing two cross circuited electrodes immersed in 0.02 per cent hydrochloric acid; they were kept so for at least forty-eight hours or until the potential between the two plates did not exceed 1-2 millivolts; pairs showing a greater discrepancy were discarded. A saturated K Cl bridge was used to eliminate the junction potential. The e.m.f. was measured with a potentiometer registering resistance, and two Leed and Northrup's mirror galvanometers were used as zero instruments, connected with each other by a mercury switch; equilibrium was established when the less sensitive galvanometer (6.4×10^{-9} amp.) showed no deflection; the circuit was then changed to the more sensitive instrument (1.75×10^{-9} amp.) and the final readings were taken. Each half-cell was standardized against potassium chloride solutions of known strength.

In the following protocols of two different standardizations made with two pairs of half-cell are given. The first gives the reading in resistance, the second in volts; the data from the first protocol may be used with a potentiometer made up from standard resistance boxes; from the second with a direct reading instrument such as Leed and Northrup's type K.

Chain used

Ag/Ag Cl/ K Cl m_1/bridge / K Cl m_2/ Ag Cl / Ag

Where m_1 and m_2 represent molor concentration

NUMBER	M_1	M_2	RESISTANCE, OHMS	TEMPERATURE
1	0.1	0.01	360.6	25.7
2	0.05	0.01	252.0	26.0
3	0.02	0.01	105.0	26.2
4	0.01	0.005	113.0	25.8
5	0.01	0.002	266.5	25.8
6	0.01	0.001	385.0	25.9

In the following series the same chain was used but the readings are given in millivolts for ready comparison with a direct reading instrument (Leed and Northrup's type K)

NUMBER	M_1	M_2	P (MILLIVOLTS)
7	0.1	0.01	56.32
8	0.05	0.01	39.89
9	0.02	0.01	17.32
10	0.01	0.005	17.40
11	0.01	0.002	40.62
12	0.01	0.001	58.30

These measurements were repeated several times with variations not exceeding 0.6 millivolts; equilibrium is usually reached within two minutes; to avoid polarization a telegraph key should be used to close the circuit.

The same electrodes and a reference half-cell Ag/AgCl/n K Cl—were used in the following experiments where the chlorine-ion concentration of gastric fluid was determined; the fluid was removed in the usual manner by means of a stomach tube and the results obtained by the electrometric method were compared with the results obtained by titration.

No. 13:
Resistance: 210 ohms
Potential of working cell: 1.48
Molar conc. by electrometric method: 0.3
Molar conc. by titration: 0.4

No. 14:
Resistance: 70 ohms
Potential of working cell: 1.42
Molar conc. by electrometric method: 0.143
Molar conc. by titration: 0.2

No. 15:
Resistance: 179 ohms
Potential of working cell: 1.42
Molar conc. by electrometric method: 0.247
Molar conc. by titration: 0.35

No. 16:
Resistance: 16 ohms
Potential of working cell: 1.46
Molar conc. by electrometric method: 0.0234

No. 17:
The (pathologic) gastric fluid from no. 16 was mixed with 0.6 n hydrochloric acid so that the molarity of the mixture was about 0..3 The potential was then measured in the usual manner:

Resistance: 193 ohms
Potential of working cell: 1.46
Molar conc. by electrometric method: 0.272

The titration does not agree closely with the electrometric value; since the titration gives the total amount of Cl present whereas the electrometric method determines Cl-ions only, there is no doubt that an appreciable amount of chlorine is present in "bound" form in gastric fluid.

A fluid bridge would be very cumbersome using this electrode in living tissue; several experiments were made to determine whether a gel bridge could fully replace a fluid bridge.

No. 18:
Ag/Ag NO_3/fluid bridge/Ag NO_3/Ag
e.m.f. = 61.8 millivolts
Potential of working cell: 1.42 temp: 27.0°
The same chain was used in connection with a K Cl Agar bridge e.m.f. = 61.7 millivolts
Potential of working cell: 1.42 temp: 27.0°

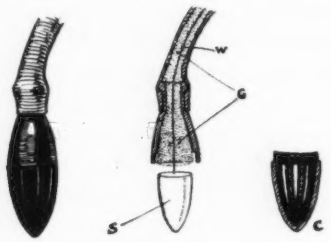

Fig 1

For investigations involving the study of changes in the chlorine-ion concentration in living tissue or fluid in body cavities an electrode of the following construction is recommended.

The container, *C*, is made from hard rubber or a similar non-conducting material and the lower end is perforated so that the body fluid under investigation may touch both the silver electrode *S* and the Agar KCl surface *G*, the latter serving as bridge. The wire, *W*, bearing a good silk and varnish insulation, makes connection with the type K potentiometer or any other potentiometer as used in the determination of hydrogen-ions; the KCl-agar in the rubber tube the latter conveniently 35 to 40 cm. long, makes connection with a calomel electrode or any other half-cell of known and constant e.m.f.; the reference half-cell should be kept at body temperature; an air bath fitted with bimetallic thermo control being sufficient as variations of $\pm$ 2° do not effect the accuracy of the method on account of the small temperature coefficient of the chain used.

BIBLIOGRAPHY

(1) NEUHAUSEN AND MARSHALL: Journ. Biol. Chem., 1922, liii, 365.
(2) AFANASJEW: Journ. Russ. phys-chem. Gesellsch., 1909, xli, 1175.
(3) BIRON: Journ. Russ. phys-chem. Gesellsch., 1908, xxxix, 1506.
(4) BRONSTED: Z. physik. chem., 1906, lvi, 677.
(5) DUNIN-BORKOWSKI: Anz. Akad. d. Wiss. Krakau, 1909, 660.
(6) GALEOTTI: Atti. R. Accad. d. Lincei, 1903, xii, 2.
(7) JAHN: Z. physik. Chem., 1900, xxxiii, 545.
(8) SACKUR: Z. physik. Chem., 1901, xxxviii, 129.
(9) GOODWIN: Z. physik. Chem., 1894, xiii, 577.
(10) KÜSTER UND THIEL: Z. anorg. Chem., 1900, xxiv, 12.
(11) NERNST: Z. physik. Chem., 1889, iv, 129.
(12) NERNST: Z. physik. Chem., 1899, iv, 161.

THE PHYSIOLOGICAL ACTION OF IONIZING RADIATIONS

III. X-Rays and Their Secondary Corpuscular Radiation

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When the eggs of Nereis limbata are exposed to x-rays and then fertilized, the process of membrane formation and jelly secretion is altered in a manner which is identical with that previously described as the result of β- or γ-radiation (1), (2), (3). The perivitelline space becomes uniformly enlarged on all sides of the egg, and the volume occupied by the secreted jelly diminishes in measure. The effect may be measured with as great precision as is attainable when β-rays are used, and consequently it has been employed in an examination of the relations between intensity, penetrating power, ionizing power and physiological effect. The resulting data throw some light upon the hypothesis that ionization is a step in the physiological changes brought about by certain types of radiation.

In developing this hypothesis we have pointed out in the preceding paper of this series (4) that a common property of the α- and γ-rays of radioactive origin, of x-rays, and of ultraviolet light—all of which produce qualitatively similar effects upon the Nereis egg—is the liberation of secondary corpuscular radiations similar in all physical aspects to slow speed β-rays. In that paper we presented some evidence which suggests that the physiological action of the α-particle may be due to such secondary radiation. In the present investigation the physiological effects of secondary corpuscular radiation excited by x-rays have been demonstrated and studied in their relation to the physical properties and physiological effects of the primary x-rays which give them origin.

Intensity of x-radiation and the velocity of physiological change. In the first paper of this series (5) it was shown that a comparison of the velocity coefficient of the physiological change in Nereis eggs produced by β-radiation, and the intensity of this radiation yielded data which could be interpreted to indicate that ionization phenomena are involved in the process, and that a considerable proportion of the ions formed by the radiation recombine without producing physiological effects. Similar experiments have consequently been carried out with x-radiation.

Our source of x-rays was a Coolidge Portable Apparatus loaned by the General Electric Company through the courtesy of Dr. W. R. Whitney. The apparatus was operated with 100 volts across the primary circuit and 5 milliamperes through the bulb. Under these conditions the bulb tends to over-heat if operated continuously for several minutes. Consequently the exposures had to be intermittent, one minute of radiation alternating with one minute during which the bulb was allowed to cool.

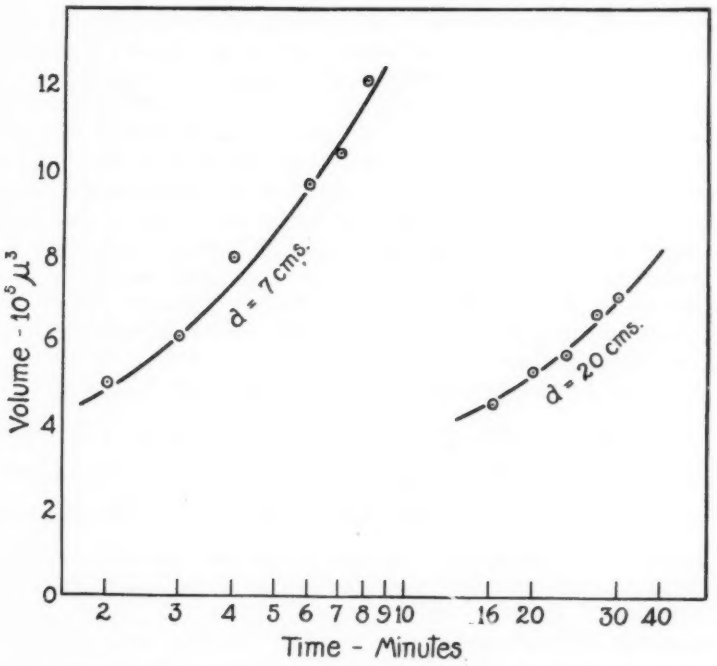

Fig. 1. Curves illustrating the influence of intensity of radiation with x-rays upon the process effecting jelly secretion of Nereis eggs. Time of radiation in minutes measured logarithmically along the abscissa. Volume of perivitelline space measured in 100,000 cubic micra along the ordinate. August 12, 1922.

This procedure is justified by the fact that the resulting change in the egg is irreversible (1). The times recorded are the sum of the periods of actual radiation. The intensity of x-rays, in all cases where the limited absorption by air may be neglected, is inversely proportional to the square of the distance from the target of the x-ray bulb. In the present experiment eggs of Nereis limbata were exposed to x-rays for varying periods of time at two distances, 7 and 20 cm. The relative intensities

were consequently 400 and 49, or 8.16:1. The eggs were placed on a thin glass plate resting on ice water during the exposure which kept them at a temperature of 0 to 2.0°C. This was done in part to prevent overheating, but chiefly because, as shown in the case of β-rays (5), a rather greater part of the ions formed in the protoplasm may be expected to recombine if the temperature is low. Figure 1 illustrates the result of such an experiment. Each point represents a determination of the average volume of the perivitelline spaces resulting from the radiation of thirty eggs. The lines drawn through the points are spaced as they should be if the velocity of the reaction is proportional to the intensity of radiation, i.e., if $k = \theta I$. It may be seen that the experimental points fall close to these lines, the discrepancy being not more than can be attributed to errors in measuring the distances. It may be concluded that *the velocity of the reaction under x-radiation is directly proportional to the intensity of the radiation.*[1] This result is of interest as it indicates that the reaction follows the general law of Bunsen and Roscoe that equal photochemical effects are produced whenever the product of intensity and time of illumination are the same. The result is of practical importance as it enables us to calculate the velocity under any intensity provided the velocity at one intensity is known—at least for intensities of the order used in this experiment.

The result is quite different from that obtained with β-rays (5) for the term $\frac{\alpha}{\beta^2} k^2$ in the equation $\frac{\alpha}{\beta^2} k^2 + k = \theta I$ has disappeared. In terms of the theory from which this equation was deduced this can occur only when $\frac{\alpha}{\beta^2}$ becomes zero, as it will do whenever the tendency of ions to recombine, measured by α, is negligible. We were at one time inclined to take this observation as evidence that no recombination of ions took place under conditions of x-radiation. This conclusion was found, however, to be incompatible with subsequent experiments. Taken by itself, the present experiment affords no evidence concerning the mechanism underlying the physiological action of x-rays.

[1] The conclusion of Kroenig and Friedrich (6) that with a difference of intensity of 1:8 equal doses do not produce like effects, but the biologic action is stronger with the high intensity acting for a short period of time, appears to contradict our conclusion. We believe a great deal of confusion will arise in the discussion of dosage unless some account is taken of reparative processes which go on during the time of radiation. Where the exposure lasts for many hours, as in the experiments of these investigators, such processes may be conceived to counteract considerably the effect of the radiation. In the case of the reaction which we have studied in the Nereis egg the change produced is irreversible and consequently this complication does not arise.

Physiological and ionizing effects of x-rays of different penetrating power. If ionization phenomena are suspected of playing a part in the physiological action of x-rays it is pertinent to inquire whether any relation exists between the velocity of the physiological change under x-radiations of different frequency or penetrating power and the ability of these radiations to ionize air. As in the case of analogous experiments carried out with α- and β-rays of different velocities, such evidence is not crucial in indi-

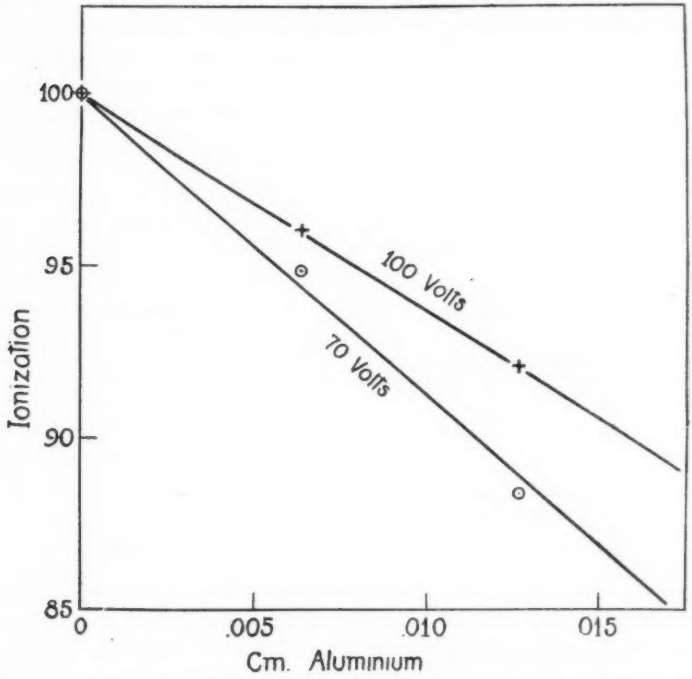

Fig. 2. Absorption of the "hard" (100 volt) and "soft" (70 volt) **x-rays** by aluminum.

cating that ionization is a step in physiological action, but such relations must be examined before any ionization theory can be upheld.

Two conditions of x-radiation have been employed. "Harder" rays were produced by operating the apparatus with 100 volts in the primary circuit and 5 milliamperes across the tube. "Softer" rays were produced with 70 volts in the primary circuit and 5 milliamperes across the tube. The penetrating power of the rays under the two conditions was examined by measuring their absorption by thin sheets of aluminum, using the apparatus described below. Figure 2 illustrates the absorption curves so

obtained, and indicates that the softer rays lose approximately 1.5 as much energy in a thin layer of matter as do the harder rays. Obviously this is not a great difference, and the experiment should be extended to rays of a greater range of penetration or frequency. This unfortunately could not be done with the apparatus available for the present investigations.

Comparison of the ionizing powers of the two qualities of x-radiation was made with an apparatus arranged as in figure 3. The flow of electricity between the plates of the ionization chamber, as the result of ionization of the air within it by the x-rays, charged one pair of quadrants of the electrometer. The condenser served to reduce and adjust the rate at which this occurred, and this enabled the sensitiveness of the apparatus

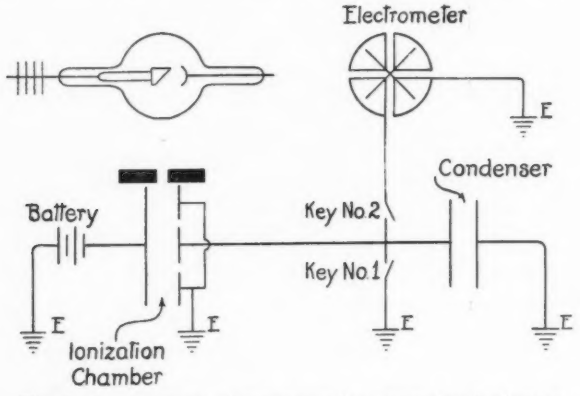

Fig. 3. Arrangement of apparatus for measuring ionization

to be controlled to suit the conditions of radiation. Since the x-ray apparatus could not be operated continuously for more than a minute without danger from overheating it was necessary to use intensities of radiation so great that the electrometer vane was not able to follow the charging of the quadrants. Consequently the method was adopted of allowing the rays to charge the electrometer for a fixed period of time and reading the total deflection produced, on a calibrated scale, after the needle had come to rest. This was done by means of the two keys. Starting with both closed, the vane at rest, and the x-ray apparatus running, charging was commenced by opening no. 1 and terminated after a measured interval by opening no. 2, whereupon no. 1 was closed again. The apparatus gave readings which checked to 1 per cent, the accuracy being limited by the difficulty of controlling the output of the x-ray apparatus. Greater accuracy was always reached in measuring radiations from radium emanation.

The design of the ionization chamber for measurements of radiations of different quality is of great importance.[2] In the ordinary types of chamber much of the ionization is due to secondary radiations arising from the walls of the apparatus. In addition a portion of the energy given up by the rays on their absorption by the air within the chamber is lost as secondary radiation which escapes from the apparatus. These phenomena affect the measurements to the same relative degree provided the radiation is of the same quality and may in this case be neglected. When the quality of the radiation is varied, however, as in the present case, the error due to these causes will not be a constant fraction of the measurement and may easily invalidate the comparative measurements. To avoid these difficulties an ionization chamber was constructed so that a small pencil of rays entered through a hole in a lead plate at one end and passed out at the other end without striking the walls in any place. In this way ionization due to secondary radiation from the walls was eliminated. In order to reduce to a minimum the loss of energy from secondary rays arising from the air it was arranged that only the ions in a thin layer of air across the middle of the chamber contributed to the measurement. This was done by placing three plates on one side of the chamber, opposite to the charged plate. Of these the middle one alone was connected to the electrometer, the other two being earthed. With such an arrangement, only those ions formed in the layer of air immediately between the middle plate and the charged plate opposite contribute to the measurement. For every secondary ray which leaves this layer of air, a secondary ray from outside may be expected to enter the layer—

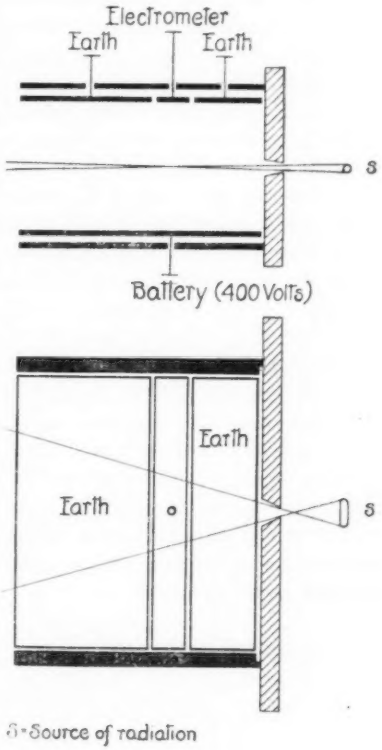

Fig. 4. Ionization chamber (see text)

[2] We wish to acknowledge invaluable advice from Dr. William Duane in this connection. The ionization chambers which we used were modeled after those in use by him at the Jefferson Physical Laboratory.

provided the chamber is sufficiently large. In order to determine whether this condition was realized measurements were made with chambers of two different sizes in order to determine whether the relative values of the measurements of the two qualities of x-radiation remained the same.

The structure of the smaller ionization chamber is illustrated in figure 4. The front is covered with a lead plate 0.5 cm. thick which completely excludes all x-rays save those which enter through a hole 0.2 cm. in diameter. This hole widens inward so that rays entering it do not impinge upon its sides. The insulated plates on the opposite sides of the chamber are separated by 3.8 cm. The charged plate is 8 cm. in height and 6 cm. in width. The three plates on the opposite side are respectively 1.9, 1.0, and 2.9 cm. wide, being separated by a distance of 0.1 cm. The middle one, which is connected to the electrometer, is thus 2.0 cm. from the front of the chamber. With these dimensions secondary rays arising in the layer of air in which the measurements are made have a free path of at least 1.8 cm. before striking the walls of the chamber.

The larger chamber was similar in design but was 9 cm. deep and 8 cm. across between the insulated plates. The plate connected with the electrometer was of the same width as before, 1.0 cm., but was placed 4 cm. behind the leaden front of the chamber. This was 1.2 cm. thick and had a hole 0.3 cm. in diameter at the outer end. With this arrangement the free path of the secondary rays arising in the middle layer of air is 4.0 cm. This chamber is of about the largest dimensions which it is practical to use, as it required over 400 volts to produce saturation.

Measurements were made of the ionization produced by the x-ray apparatus when run with 100 volts and with 70 volts in the primary circuit—the distance between the target and the front of the ionization chamber being kept constant and approximately 20 cm. The results tabulate as follows:

	RELATIVE IONIZATION BY X-RAYS	
	70 volts 5 milliamps.	100 volts 5 milliamps.
Small ionization chamber	1.00	2.00
	1.00	2.03
	1.00	2.12
Large ionization chamber	1.00	2.14
	1.00	2.14
	1.00	2.16
	1.00	2.13

It appears that with the machine run at the higher voltage the ionization, when measured with the large chamber, is 2.15 times that produced at the

lower voltage. The ratio is about 5 per cent lower when the smaller ionization chamber is used, which indicates that this vessel is too small to include all the secondary radiation and throws some doubt on the adequacy of the larger vessel. It is clear, however, that the size of the vessel has not had a great effect on the relative value of the measurements, and consequently the results may be taken as a pretty exact measurement of the relative ionizing powers of the x-rays in question.

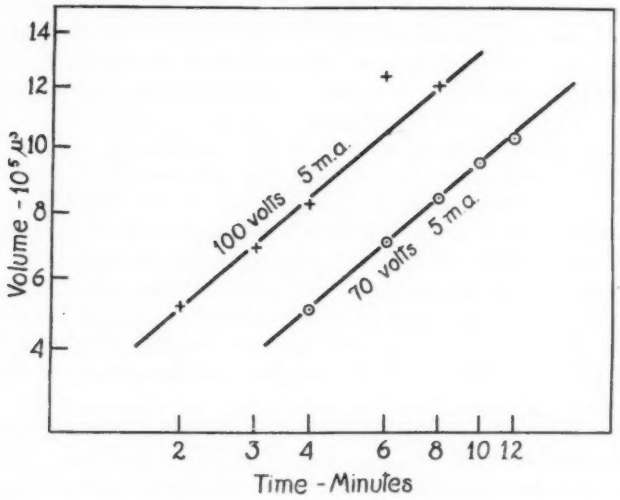

Fig. 5. Curves illustrating the relative effects of "hard" (100 volts) and "soft" (70 volts) x-rays upon the process effecting jelly secretion of Nereis eggs. Time of actual radiation in minutes measured logarithmically along abscissa. Volume of perivitelline space—measured in 100,000 cubic micra along ordinate. August 17, 1922.

Turning to the comparison of the physiological effectiveness of the two qualities of x-rays data are shown in figure 5 which were obtained by exposing Nereis eggs to each for varying periods of time. The exposure was intermittent, the bulb being alternately operated for one minute and then allowed to cool for one minute. The distance between the target and the eggs remained fixed at 7 cm. throughout. The eggs rested on a glass plate the under surface of which was in contact with iced water. The points shown are experimental determinations of the volume of the perivitelline space resulting from radiation, and are each based on the average of measurements of thirty eggs. The lines drawn through the points are spaced on the assumption that the velocity of the reaction is twice as great with 100 volts as with 70 volts. It appears from this that the relative physiological effectiveness of the two qualities of radiation

differs from the relative ionizing power as 2.0 differs from 2.15, or by about 7 per cent. This is an excellent agreement when the difficulties of the comparative measurements are considered, and fulfils satisfactorily the requirements of the theory that ionization is a fundamental step in physiological action.[3]

The secondary corpuscular radiation induced by x-rays. When x-rays are absorbed by matter, secondary rays are generated which have the absorbing matter as their source. Part of the secondary radiations are x-rays, and as such the interpretation of their physiological effects introduces no problems different from the considerations to which the action of the primary radiation gives rise. The remainder of the secondary radiation is in the form of negatively charged corpuscles, or electrons, which may be considered identical in their properties with slow-speed β-rays. It is now generally accepted that the ionization produced by x-rays is due solely to these corpuscular rays (7). It follows from this, as a consequence of the theory here developed, that the physiological effect of x-rays must also be due to the secondary corpuscular radiation. The theory receives strong support from the following experiments which demonstrate that physiological effects can be produced by such radiation, and that certain correlations exist between the physiological effects of primary and secondary radiation and the ionization set up during their absorption.

When x-rays fall on metallic radiators the intensity of the corpuscular emission increases with the atomic weight of the radiator. Favorable conditions for the exposure of eggs to such radiation are obtained by placing them on a gold plate and causing the primary beam of x-rays to traverse the eggs and set up corpuscular radiation in the gold surface on which they rest. When this is done and the eggs are subsequently fertilized, it is found that the perivitelline space is markedly enlarged on one side, and that the jelly is diminished in proportion on the same side. The result resembles that produced by radiation of limited penetrating power such as α-particles or ultra-violet light. The distribution of the enlarged area of the perivitelline space is more limited than is usual with those radiations, and indicates that the rays which have produced it do not penetrate to more than a quarter of the diameter of the egg, that is, to about 0.003 cm. This range is concordant with the known properties of the corpuscular rays which are stopped for the most part by a centi-

[3] The conclusions of Kroenig and Friedrich (6) are concordant with this result so long as the quality of the rays does not differ greatly. When this occurs they find that the more penetrating radiation has a greater biological action than is to be anticipated from the ionization measurements. We believe this is due chiefly to the failure of their ionization chamber to account for differences in the secondary radiations set up in its walls. It may also be due in part to factors which are discussed in footnote on page 374 of the following paper.

meter or so of air. Control experiments demonstrated that the effect is not due to the gold in the absence of radiation, and that the phenomenon is not present in detectable degree if the eggs rest on a material of lesser density such as glass.

The action of the corpuscular rays from the gold radiator is seen to be of singular interest when it is considered that these radiations must be identical in their properties to the corpuscular rays excited within the protoplasm of the egg, for it has been demonstrated by Sadler (8) that the penetrating power (and hence all the other variable properties which are related to the velocity) of the corpuscles is independent of the nature of the radiating material, and depends solely on the quality of the exciting x-rays.

If the action of x-rays is due to the secondary corpuscles it follows that a direct correlation should exist between the physiological effects of the primary beam and the effects of the secondary corpuscles from the gold radiator. Since Innes (9) has demonstrated that the velocity of the corpuscles is independent of the distance of the x-ray bulb from the radiator, it is clear that when the intensity of the primary beam is varied by altering this distance the quality of the secondary radiation is unchanged, but the number of corpuscles is altered in proportion to the change in intensity. Consequently the same relation should exist between the effects of the primary and secondary radiation, when the distance is great as when the distance is small.

In the Nereis egg which has been exposed to secondary corpuscular radiation from a gold plate, the localized enlargement is due to the corpuscular rays plus the primary x-rays which have traversed the egg before reaching the gold plate. The membrane on the opposite side of the egg has not been reached by an appreciable quantity of corpuscular rays; effects upon it are due to the primary beam alone. The same egg may consequently be used to measure the relative effects of primary and secondary radiation.

Figure 6 illustrates the result of an experiment in which small drops of sea water containing Nereis eggs were placed on a gold plate chilled with ice and exposed to x-rays for various periods of time at distances of 7 and 20 cm. (relative intensities 8.16:1). The x-ray bulb carried 5 milliamperes, the voltage in the primary circuit being 100 volts throughout the experiment. In this figure the effect of the primary x-rays is plotted as in a correlation table against the effect of the corpuscular radiation for various times of exposure. It may be seen that the relation is the same whether the intensity be high (7 cm. distance) or low (20 cm. distance). The difficulties which cause the measurement of α-ray effects to be rather unprecise (2) are encountered in measuring the effects of the secondary corpuscles and account for the rather great variation which

the results show. The variations occur at random, however; consequently the result may be taken to indicate that the relative effects on the opposite sides of the egg are approximately the same irrespective of the intensity of the exciting radiation.

The implication of this result is that the physiological effect of the secondary radiation varies with the number of corpuscles excited in exactly the same way as the effect of the primary beam varies with its

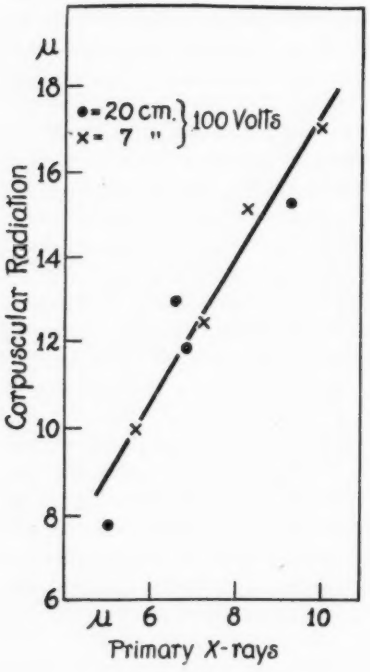

Fig. 6

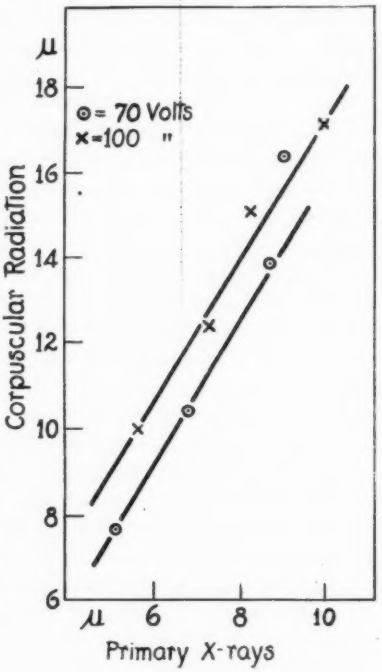

Fig. 7

Fig. 6. Correlation between effect of the corpuscular radiation and the effect of the primary beam of x-rays compared when the latter is of "high" and "low" intensity.

Fig. 7. Correlation between the effect of the corpuscular radiation and the effect of the primary beam of x-rays compared when the latter is "hard" (100 volts) and "soft" (70 volts).

Each point in figures 6 and 7 represents the average measurement of a lot of 15 eggs. The different points represent exposures of different duration. The thickness of the perivitelline space on the side of incidence of the primary x-rays is measured in micra along the abscissa; the thickness on the side of incidence of the corpuscular rays is measured along the ordinate. August 21, 1923.

intensity. The latter case has been seen to follow the law $k = \theta I$, where k is the velocity coefficient of the process. For the corpuscular radiation we may consequently write $k = \phi N$ where N is the number of corpuscles, thus formulating the proposition that the velocity coefficient of physiological change resulting from corpuscular radiation is proportional to the number of corpuscles excited.

If the physiological effects of x-rays are due to ionization established by the secondary corpuscular radiation, the relative effects of the primary beam and the secondary radiation from the gold plate should be the same as the relative ionization set up by these rays respectively in a thin layer of air. A somewhat crude experiment demonstrates this to be the case. From the data on which figure 6 is based it appears that to produce equivalent physiological effects the primary beam alone must act approximately four times as long as the combined primary and secondary radiation. The effective intensity of the combined primary and secondary radiation is therefore four times that pf the primary beam. The relative ionizing powers of the primary and secondary radiations have been measured with the aid of the ionization chamber illustrated in figure 4. After determining the amount of ionization produced in a layer of air one centimeter deep by a primary beam of x-rays of the same quality as used in the physiological experiment, a gold plate was placed in the path of the primary beam just as it emerged from the layer of air in which the ionization was measured. When the amount of ionization formed in the layer as the result of the combined action of the primary and secondary radiation was now measured it was found to be 4.2 times as great as when the primary beam alone was acting on the air. The agreement between the relative physiological effectiveness and the ionization produced by primary and secondary radiation is very much better than the conditions of the experiments warrant one to expect. The quantitative agreement cannot be stressed. It is proper to conclude, however, that the *general magnitude* of the ratio between the physiological effects of the primary x-rays and the secondary corpuscles which they excite is the same as their relative ionizing powers.

The velocity of the secondary corpuscles was shown by Innes (9) to decrease as the potential applied to the x-ray bulb used in exciting them was diminished. We may inquire whether the relation between the effectiveness of the primary and secondary corpuscular radiation remains the same when the velocity of the latter is varied in this manner. The preceding experiment was repeated, this time with a distance of 7 cm. between the eggs and the target of the x-ray bulb, a current flow of 5 milliamperes, but with the voltage applied to the primary circuit at 100 volts in one series of observations, at 70 volts in the other. We have seen that with the apparatus operated at 100 volts the primary x-rays

are twice as effective and have approximately 1.5 times the penetrating power, as compared to the rays generated when 70 volts are employed in the primary circuit. The secondary corpuscles may be expected to be of lower velocity in the latter case. Figure 7 illustrates the result of the experiment. With the exception of one point, which owes its discrepancy to one of the sixteen measurements on which the figure is based, the data show that for equivalent effects due to the primary beam, the additional effect due to the secondary corpuscles from the gold radiator is less when the exciting rays are generated by the machine run at the lower voltage. This conclusion appears at first sight to be in contradiction to the expectation that a direct correlation should exist between the effects of the primary beam and those of the secondary corpuscles radiated from the gold plate. The discrepancy is to be expected, however, for the secondary rays excited at the lower voltage will be of lower velocity and hence of less penetrating power than those excited at the higher voltage. Consequently, relatively fewer of them will escape from the gold and enter the egg, and those which do so will have given up a larger part of their energy in the process. That this is actually the case is shown by a measurement of the increased ionization produced by introducing a gold plate in the path of the primary beam of x-rays. We have seen that in the case of the beam excited with 100 volts across the primary circuit the ionization was increased 4.2 times by the addition of the secondary radiation. When the measurement is repeated with a voltage of 70 the increase is only 3.4 fold. For equivalent intensities of primary radiation the secondary radiation which actually escapes from the radiator when the lower voltage is employed is only about three-fourths the amount which is liberated at the higher voltage. When the conditions are understood, the apparently contradictory experiment becomes a striking confirmation of the theory, for it shows that when the conditions are altered so as to reduce the relative ionizing powers of the secondary corpuscular radiation the relative physiological effects are reduced also.

SUMMARY

1. Over a range of intensity of 1:8 the velocity of the reaction under x-rays is directly proportional to the intensity of the radiation.

2. X-rays differing in their penetrating power as 1.0:1.5, agree in their relative ionizing power and relative physiological effectiveness within about 7 per cent.

3. Secondary corpuscular radiations induced during the absorption of x-rays by matter produce physiological effects similar in quality to those of the primary x-ray beam.

4. These secondary corpuscles have a range in protoplasm of approximately 0.003 cm.

5. The physiological effect of the secondary radiation varies directly with the number of corpuscles emitted.

6. The general magnitude of the ratio between the physiological effects of the primary x-rays and the secondary corpuscles which they excite is the same as their relative ionizing powers. When the quality of the primary x-rays is altered the relative physiological effects and ionizing powers of the secondary corpuscles are varied in the same direction.

BIBLIOGRAPHY

(1) Redfield and Bright: This Journal, 1918, xlv, 374.
(2) Redfield and Bright: Journ. Physiol., 1921, lv, 61.
(3) Redfield and Bright: Journ. Gen. Physiol., 1919, ii, 25.
(4) Redfield and Bright: This Journal, 1924, lxviii, 62.
(5) Redfield and Bright: This Journal, 1924, lxviii, 54.
(6) Kroenig and Friedrich: The principles of physics and biology of radiation therapy. (Trans. H. Schmitz), New York, 1922.
(7) Kaye: X-rays. London, 1917.
(8) Sadler: Phil. Mag., 1910, xix, 337.
(9) Innes: Proc. Roy. Soc., London, A, 1907, lxxix, 442.

THE PHYSIOLOGICAL ACTION OF IONIZING RADIATIONS

IV. Comparison of β- and X-Rays

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Knowledge of the precise factors which determine the relative effectiveness of different kinds of radiation is required not only for the understanding of the mechanism of their action, but for the use of the therapeutist who must choose between the various sources of radiation at his disposal. As a result of the work of Grotthus and Draper it has become clearly understood that radiation must be absorbed in order to produce an effect. The absorption coefficient is consequently the factor of prime importance in determining the effectiveness of any kind of radiation. It does not necessarily follow, however, that all the absorbed energy contributes directly to physiological change and the factors which determine what fraction will do so are for the most part unknown.

Our experiments upon the Nereis egg have provided unequivocal evidence that some factor other than the intensity and the absorption coefficients of radiation determine the relative magnitude of the physiological changes produced when rays of different quality act on similar living tissues. If this were not the case when equal quantities of energy were absorbed by similar masses of protoplasm from two dissimilar sources of radiation in a given time as the result of proper adjustment of the intensities in relation to the absorption coefficients, then equal physiological effects should be produced not only at these intensities, but under any condition in which the relative intensities of the two types of radiation remain the same. This follows from the consideration that the absorption coefficients of radiations do not vary with the intensity of the radiation. If the relation between intensity of radiation and the velocity of the resulting physiological change in the case of the Nereis egg be compared when β-rays and x-radiation are employed it is clear that these conditions are not fulfilled. In the case of x-radiation the relation is a linear one described by the equation $k = \theta I$ (1). When β-rays are employed the relation is described by the equation $\frac{\alpha}{\beta^2} k^2 + k = \theta I$ which

is graphically represented by a curve concave toward the coördinate along which the intensity is measured (2). The relation may be pictured as in figure 1 in which OX represents the variation in the velocity of the reaction when the intensity of x-radiation is varied and OB represents the corresponding relation for β-radiation. It is clear from this figure that intensities can be chosen, as at i_2, at which equal physiological effects are produced by the two kinds of radiation. If, however, the intensities are each halved, as at i_1, it is seen that the β-radiation becomes more effective than the x-radiation. If the intensities are each increased

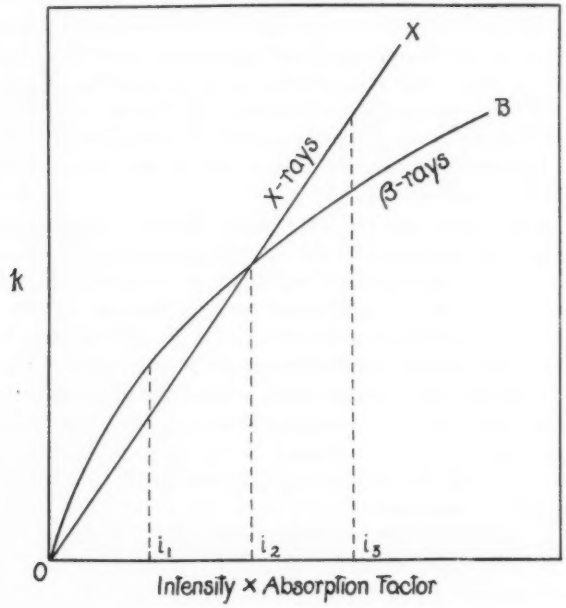

Fig. 1. See text.

50 per cent, as at i_3, the x-radiation surpasses the β-radiation in its effects. Whatever the nature of the factor responsible for this difference in the action of different kinds of radiation may be, it must be taken into account by the practical radiologist in his consideration of dosage. The present paper is a consideration of the nature of this factor.

If the observations described in a preceding paper (2) on the action of β-rays be accepted as indicating that ionization is a step in physiological action, and that many of the ions formed may recombine without producing physiological effects, it follows *that the relative effectiveness of different kinds of radiation depends on the coefficients of recombination of*

the ions formed in the protoplasm, as well as upon the coefficients of absorption of the radiations and their intensities. To put the foregoing conclusion to experimental test it should be shown that equal physiological effects are produced by dissimilar sources of radiation when their ionizing powers and the tendency of the ions to recombine are the same, or when inequalities in ionizing power are exactly compensated for by differences in the tendency for recombination to occur. In the following experiments made with β- and x-rays it has proved possible to demonstrate in a rough way the equality of physiological effect under conditions of equal ionizing power and equal tendency for recombination.

The physiological response of the egg of Nereis limbata to β- and x-rays can be measured with sufficient precision to warrant an attempt at a comparison of the relative physiological and ionizing effects of these radiations. While the results are far from satisfactory they are presented because they appear to be the most precise determinations yet made, and in order to point out the various factors and sources of error which must be considered in such a comparison.

By exposing Nereis eggs from the same parent to both x-rays and β-rays of various intensities and for various periods of time it has been determined in two independent experiments that equal physiological effects were produced by β-radiation at an intensity of 30 millicurie centimeters for 15 minutes at a temperature of 20 to 22°C., and by x-radiation from a Coolidge Portable Apparatus with 98 volts in the primary circuit, 4.7 milliamperes flowing through the tube at a distance of 7 cm., acting for 4.8 minutes at a temperature of 0 to 2°C. Since the velocity of a process is inversely proportional to the time required to produce a given effect the relative velocities of the physiological actions under these standard conditions were

$$\frac{\text{Velocity under x-radiation}}{\text{Velocity under } \beta\text{-radiation}} = \frac{15}{4.8} = \frac{3.12}{1}$$

Taking the temperature coefficient for 10°C. as 1.1 (a procedure justified in the following section) and allowing for the difference in temperature at which radiation took place, the ratio becomes

$$\frac{1.1^2 \times 3.12}{1} = \frac{3.77}{1}$$

The relative ionizing powers of these two standard conditions of radiation were measured with the methods and apparatus used in comparing x-rays of different penetrating power (1). Using the larger ionization chamber the ratio of the ionizing effect of the x-rays to β-rays of the standard intensities used in the physiological experiment was 4.9:1. This result is of the general magnitude of the physiological ratio—the difference might even be explained as due to the slightly greater tendency toward recombination, which will be shown to characterize the ions produced with x-rays.

Such conclusions, however, are not entirely warranted when the sources of error are considered. The chief difficulty arises from the different nature of the secondary radiations set up within the ionizaton chamber by β- and x-rays. When the smaller ionization chamber is used in the comparison the ratio becomes 2.7:1 instead of 4.9:1. The size of the chamber, by encompassing a greater or less quantity of the secondary radiation, has a very great effect on the value of the ratio. While it is probable that the ratio obtained with the larger chamber is the more reliable, its accuracy has not been evaluated and cannot be until a practical method is devised for working with still larger chambers. The result must be taken merely as indicating agreement in the general magnitude of the relative ionizing and physiological effects of β- and x-rays.

The tendency of the ions which may be formed by x-rays in the Nereis egg to recombine, cannot be demonstrated by the method employed in the case of β-rays for reasons which will appear later. However, if it be assumed that the ionization fields produced within protoplasm are similar to those produced in air by radiation, save for a difference in scale due to the different density of the media, one may use data obtained from an ionization chamber to determine whether there is a marked difference in the tendency of ions to recombine, when set up by x-rays or by β-rays. The test consists in determining the voltage which must be applied to the plates of an ionization chamber in order to remove any given fraction of the ions formed between them by each kind of radiation when the total number of ions formed by each is the same and when the intensity is approximately equivalent to that employed in the physiological experiment.

The ionization chamber used is shown in figure 2. It consisted of a metal box 1 cm. wide, 8 cm. high and 7 cm. deep. Rays entered it through a slit in a lead plate 1.2 cm. thick which covered the narrower side. The slit was 2 cm. long and 0.2 cm. wide at the outside and was made with flaring sides so that no rays entering it from a distance fell upon the surfaces. On one side of the box there was an insulated plate which could be charged by connection to a battery of storage cells; on the other side were three insulated plates. The outer and inner ones were earthed; the middle one was connected to the electrometer. The latter plate was 1 cm. wide, its edge being 2 cm. from the lead plate. In all other respects the arrangement was similar to that described on page 358 of the preceding paper (1). The object sought in designing the instrument was to fill the space within which the ions are measured with as homogeneous an ionization field as was possible. The source of β-radiation was a glass tube 1 cm. long containing 167 millicuries of radium emanation placed 2 cm. in front of the opening of the ionization chamber. The x-ray bulb was placed 12.2 cm. from the chamber, and when operated

at 100 volts and 5 milliamperes produced an equal number of ions within the chamber. The result illustrated in figure 3 shows that the curves obtained with the two kinds of radiation agree closely in regard to the number of ions which fail to recombine before being removed from the chamber by any given voltage. Actually slightly higher voltages are required to prevent a given degree of recombination with x-rays than with β-rays. This fact may be interpreted to indicate that there is a slightly greater tendency for the ions set up by x-rays to recombine.

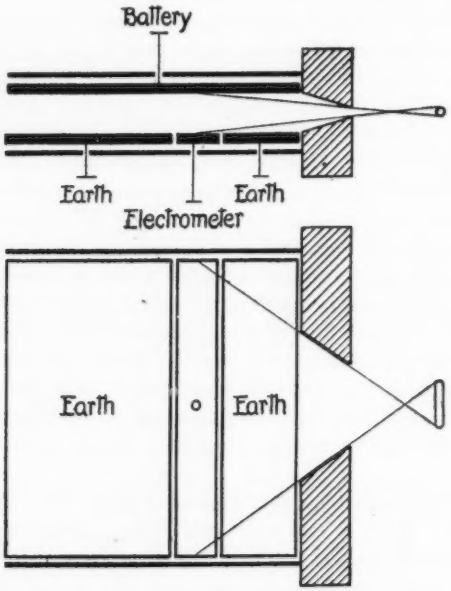

Fig. 2. Ionization chamber for measuring the recombination of ions formed by β- and x-rays. See text for description.

We are now in a position to interpret the difference in the relation of intensity of x- and β-radiation to the velocity of the physiological change which they set up in the Nereis egg. It has been shown that the effect of intensity upon the velocity of physiological action in the case of β-radiation is expressed by $\frac{\alpha}{\beta^2} k^2 + k = \theta I$ (2) and by $k = \theta I$ in the case of x-radiation (1). The difference is that the term $\frac{\alpha}{\beta^2} k^2$ which depends on the relative tendency of ions to recombine (α) or to contribute to the reaction (β) has disappeared in the latter case. This observation was considered

at first to indicate that no recombination of ions occurred under conditions of x-radiation. The foregoing comparison of the tendency of ions produced by the two kinds of radiation to recombine has forced us to abandon the view that recombination of ions does not occur when physiological effects are produced in the Nereis egg by x-rays. The disappearance of the term $\frac{\alpha}{\beta^2} k^2$ in the expression describing the effect of intensity on the velocity of reaction under x-radiation must and can be explained in another manner. If the ionization due to x-rays is produced by the sec-

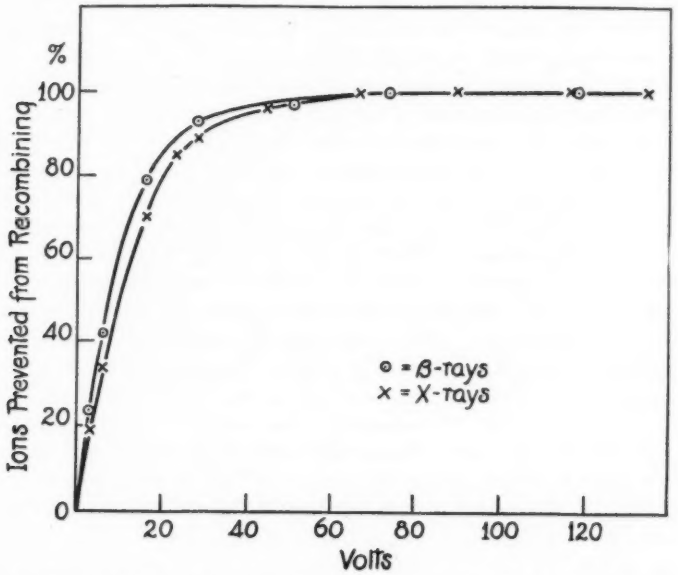

Fig. 3. Curves showing the per cent of ions formed by β and x-radiation respectively which are prevented from recombining by charging the plates of the ionization chamber to various potentials.

ondary corpuscles it is quite conceivable that the intensity may be n-creased many fold without appreciably increasing the proportion of recombinations which occur. Consider that the ions are formed in the linear path of the secondary corpuscle, as is indicated in the preceding paper (1). In recombining they may unite with other ions from the same corpuscular path or with ions from other corpuscular paths. If the intensity of the exciting x-rays is low the chances are greatly in favor of recombination within the same corpuscular path for *a*, the corpuscles being of low velocity the ions will be formed close together, *b*, the x-ray

wave front being diffuse will set up corpuscles distributed in space at random; the chances of their paths being close together are consequently small. Recombination is consequently dependent on the density of the ions in each corpuscular path rather than on the density of the paths themselves. If the intensity of the exciting x-rays is increased the number of corpuscular paths is increased but the character of the ionization field of each remains unchanged. There will be exactly the same tendency to recombination within each path; the chances of recombination between paths will be increased in measure with the intensity of the primary beam. But if the chances of such recombination are negligible to begin with this form of recombination will not alter the proportion of ions recombining until the intensity is increased very greatly. Consequently if the corpuscular paths are not too dense a change in the intensity of the exciting rays will change the number of ions formed without altering appreciably the proportion of them which recombine. The resulting physiological change will consequently vary in velocity in proportion to the variation in intensity of the primary beam, as we have seen to be the experimental fact. We have also demonstrated that the velocity coefficient of the physiological effect of the secondary corpuscular radiation is at least roughly proportional to the number of corpuscles emitted.[1]

If the foregoing conclusions hold for x-rays why do they not also apply to the β-ray phenomena? The β-rays possess a much higher velocity than the secondary corpuscles set up by x-rays, consequently the ions within the path of a β-ray may be conceived to be distributed much farther apart than in the path of a secondary x-ray corpuscle. To produce equal numbers of ions per unit volume the number of β-rays traversing the volume must be much greater than the number of secondary corpuscles from x-rays. Consequently the chances of recombination of ions between paths is much larger in the case of β-radiation.

The picture of the mechanism underlying the physiological action of x-rays which is drawn in the preceding section receives support from a determination of the temperature coefficient of the reaction. It has been shown that the velocity coefficient, k, of the reaction is proportional to the intensity, I, of x-radiation, or $k = \theta I$. On the assumption that recombination of ions does not occur, which is one interpretation of this relationship, the limiting factor determining k is the rate at which ions are formed under unit intensity or θ. We have seen on general theoreti-

[1] This picture of the ionization fields set up by x-rays in protoplasm affords an explanation of the belief held by radiologists that γ-ray are more effective physiologically than x-rays of equivalent ionizing power (3). The secondary corpuscles excited by the former will have a higher velocity than those excited by the latter. The ions formed in their paths will consequently be farther apart and there will be less tendency for recombination to occur.

cal grounds, confirmed by direct experiment in the case of the action of β-rays on Nereis eggs, that this term is unaffected by the temperature under which radiation takes place (2). Consequently if this interpretation of the relationship is correct, k should be uninfluenced by the temperature—the temperature coefficient,[2] Q_{10}, should be 1.0.

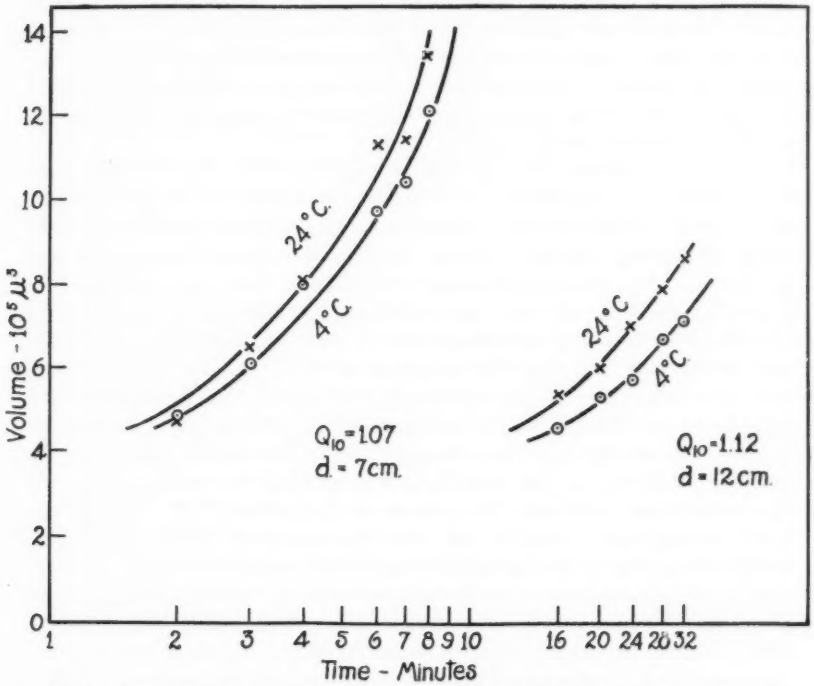

Fig. 4. Curves illustrating the effect of exposing Nereis eggs to different intensities of x-radiation at 24°C. and at 4°C. for various periods of time. Time of exposure measured logarithmically in minutes along the abscissa; the volume of the resulting perivitelline space, each point based on the average of measurements of 30 eggs, measured in 100,000 cubic micra along the ordinate August 12, 1922.

The alternate interpretation is that ionization and recombination take place in the paths of the individual secondary corpuscles, and that altering the intensity of radiation alters the number of paths but not the rela-

[2] Calculated from the equation of Kanitz (4)

$$Q_{10} = \left(\frac{k_2}{k_1}\right)^{\frac{10}{T_2 - T_1}}$$

where k_2 is the velocity coefficient at temperature of T_2 and k_1 is the velocity coefficient at temperature of T_1.

tive numbers of ions which recombine or contribute to the reaction within each path. In the case of the action of β-rays we have shown that temperature has a definite effect upon this ratio, with the result that the temperature coefficient, Q_{10}, of the velocity constant, k, has a value ranging between 1.1 and 1.3 (2). It should follow if this interpretation is correct that the temperature coefficient under x-radiation should be of this magnitude. Further, since the density of the ionization field in the path of each secondary corpuscle will be unchanged by an alteration in the intensity of radiation, it should follow that the ratio of recombination to reaction will be influenced by a given temperature change to an equal extent at different intensities of radiation.

Figure 4 illustrates the result of an experiment in which Nereis eggs were exposed to x-radiation at two different temperatures, 4° and 24°, for varying periods of time. The exposures were intermittent, the bulb being alternately operated for one minute and allowed to cool for one minute. In one series the distance between the target and the eggs was 7 cm., in the other 20 cm., the corresponding ratios of intensity being 1:8.16. The lines drawn through the experimental points are spaced on the assumption that Q_{10}, the temperature coefficient for 10°C. is 1.07 and 1.12 respectively. The agreement is close enough to warrant the acceptance of these values as experimental determinations. It may be concluded consequently that temperature has a definite effect on the velocity coefficient of the reaction resulting from x-radiation, and that the temperature coefficient has a value of the order of 1.1. The experiment consequently confirms the view that ionic recombination occurs within the paths of the individual secondary corpuscles.

The coefficients obtained with the two intensities do not differ by more than may be attributed to experimental errors. They consequently conform to the expectation that the intensity of radiation will not influence the value of the temperature coefficient. It is desirable to know whether this condition holds over a greater range of intensities in order to obtain more crucial evidence on this point.

ADDENDUM. In presenting as extensive and varied a series of data as that contained in the papers of this series it is desirable to attempt some evaluation of the whole. The fundamental conception toward which all the experiments point is that radiation produces in living matter ionization phenomena essentially similar to those known to occur in gases and liquids, and that physiological action depends qualitatively and quantitatively upon such ionization. Such a conception can scarcely be regarded as proved until the ions formed within protoplasm are measured and their quantity related to the magnitude of physiological action. Lacking the ability to do this, recourse has been had to less direct methods of correlating the physical properties of radiation and their physiological

characteristics. The variety of phenomena which have been correlated by the theory in this way is sufficiently great to warrant a considerable degree of faith in its adequacy, provided the experimental determinations be deemed reliable and the assumptions underlying the reasoning are correct. With regard to the observations we believe that the reaction which we have studied is capable of more precise quantitation than any heretofore employed in similar studies; and in the course of the several years through which the investigations have extended, our methods of handling the biological material have been thoroughly tested and standardized. The assumptions which have been made have been concordant throughout with the simplest expection on the ground that the ionization effects within the cell are comparable to those occurring in better known physical systems. The fact that the conclusions are in every respect such as might be drawn from preëxisting physical conceptions is perhaps the strongest evidence in their favor. We present these results, however, not because we believe they prove an hypothesis, but because they set up a series of conceptions concerning the mechanism of the physiological action of ionizing radiations definite enough to demand examination by those who may devise better methods and more crucial experiments.

Concerning the general application of the theory it is curious that the effects of recombination of ions have not made themselves apparent in the case of purely chemical processes, proceeding under the influence of radiation. The explanation probably lies in the fact that such reactions are carried on in homogeneous systems in which the components are thoroughly mixed. In a polyphasic system such as protoplasm it is possible that the time required for the diffusion of the products of ionization to phase boundaries where reactions occur may be sufficiently great to give an opportunity for ionic recombination to occur.

It is our view that ionization phenomena can be held to be important only in the case of action of the so-called ionizing radiations, i.e., α-, β-, γ-rays of radioactive origin, x-rays and ultra-violet light. In extending the view that such phenomena are responsible for the physiological effects of light of greater wave length than the ultra-violet, great reserve must be exercised. The expulsion of electrons from green leaves and chlorophyl by ultra-violet light has been demonstrated by McClelland and Fitzgerald (5), but Dixon and Poole (6) have been unable to obtain this result with the wave lengths actually effective in producing photo-synthesis. Recent interesting discussions of color vision by Joly (7) and Clark (8) assume the photoemission of electrons by retinal elements. Poole has been unable, however, to demonstrate the expulsion of electrons either from the retina or its pigments under the action of light (9). Furthermore, in the field of photo-chemistry there is evidence that chemical reactions may be brought about without measurable ionization (10).

SUMMARY

1. The relative effectiveness of β- and x-rays depends on some factor additional to the intensities and absorption coefficients of these radiations.

2. This factor is considered to be the degree to which the ions formed by the various radiations tend to recombine. It is shown that when this tendency is the same for β- and x-rays the relative physiological effects and ionizing powers are also of the same general order.

3. Characteristics of the relation between intensity of radiation and the velocity of the physiological reaction under x-radiation suggest that the effects are due to the formation of dense ionization fields in the paths of the secondary corpuscles. This conception explains observed differences in the action of β-, γ-, and x-radiation.

4. The temperature coefficient of the physiological change under x-radiation is 1.1.

BIBLIOGRAPHY

(1) Redfield and Bright: This Journal, 1924, lxviii, 354.
(2) Redfield and Bright: This Journal, 1924, lxviii, 54.
(3) Kroenig and Friedrick: The principles of physics and biology of radiation therapy. (Trans. by H. Schmitz.) New York, 1922.
(4) Kanitz: Die Biochemie in Einzeldarstellungen; Temperatur und Lebensvorgänge. Berlin, 1915, part 1.
(5) McClelland and Fitzgerald: Proc. Roy. Irish Acad., 1916–17, A, xxxiii, 1.
(6) Dixon and Poole: Proc. Roy. Dublin Soc., 1920, xvi, 63.
(7) Joly: Phil. Mag., 1921, xli, 289.
(8) Clark: Journ. Opt. Soc. Amer., 1922, vi, 813.
(9) Poole: Phil. Mag., 1921, xli, 347.
(10) Thomson: Proc. Cambridge Phil. Soc., 1912, xi, 90.

BLOOD PRESSURE IN RATS UNDER DEFICIENT DIETS

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Introduction. Despite the great volume of work that has been reported during the last decade in the vitamin field, only a few scattered references have been found in the literature dealing with the relationships of vitamin deficient diets to the blood pressure in experimental animals. Although observations on the effect of vitamin deficiency or even sufficiency with reference to the heart muscle itself or parts of the circulatory system have been noted and analyzed by a limited number of workers, a search for actual quantitative data on blood pressure variability in deficient animals has so far been unfruitful.

Uhlmann (1) reported an investigation of the physiological effect of commercial preparations of vitamin B, when administered subcutaneously, intravenously and by mouth in rabbits and dogs. Among other results he noted a lowered heart action, a dilatation of the blood vessels and a lowered blood pressure. He used a mercury manometer, with magnesium sulphate as an anti-coagulant, and inserted a cannula in the carotid artery. In dogs the drop in pressure on vitamin B injection, as given in his tables (p. 477), amounts roughly to from 30 to 50 per cent, (i.e., from 90 to 70 mm. Hg) in some cases, and from 100 to 55 mm. Hg in others. In rabbits the range was somewhat greater, i.e., in one case from 95 to 35 mm. Hg. He indicated that such a preparation gave results similar to pilocarpine; that it acted as a strong vagus stimulant (inhibitory), and gave severe vasomotor dilatation, resulting in lowered blood pressure. In the light of recent investigations, however, it may be questioned as to whether his preparations should be considered as vitamin B preparations.

A little later McCarrison (2), working with animals on B-deficient diets, recorded the fact of marked atrophy of the heart muscles in pigeons, guinea pigs and monkeys, which findings he contrasted later (2 b) with those of Ellis, in human beri-beri subjects where hypertrophy seems to be the rule. Although these observations were recorded, no comment was made to indicate their possible bearing upon the maintenance of proper blood pressure in these animals. Cowgill (3) in his analysis of

vitamin B-deficiency in his dogs says, "they showed paralytic symptoms . . . . when they exerted themselves to leave their cages, they were seized with spasms and suddenly died. Heart failure was considered to be the cause of death." In conversation with one of us he states that up to the present he has made no attempt to follow blood pressure changes in his dogs.

That the heart muscles of animals contain normally considerable amounts of both A and B vitamins, when compared to skeletal muscles and other parts, was indicated in early work by McCollum and Davis (4), and was subsequently confirmed in relation to vitamin B by Osborne and Mendel (5) and later (6) for the fat soluble vitamin. By feeding animal tissues as the sole source of water-soluble vitamin for growth in rats, they showed that skeletal muscles are relatively poor in this vitamin, while heart muscle, liver, kidney and brain contain it in greater proportions. In view of more recent work by the last investigators (6) on the possibility of the storage of these vitamins in the various tissues of the body, especially in the heart, these facts become of considerable significance in relation to the maintenance of proper blood pressure. From the evidence cited by Sherman and Smith (7) there is reason to believe that animals, when put upon deficient diets, begin to draw upon their reserve supply from various organs. Gradually these stores may be mobilized to meet the needs of vital metabolic reactions and since the heart muscles would suffer from such depletion, along with the other vital organs, it seems reasonable to infer that blood pressure could no longer be maintained at its normal level.

In view of the fact that so far as we have been able to learn, no quantitative work has been done on the blood pressure of rats when placed upon diets deficient in vitamins, it is thought that the data cited in the following pages may be of some interest.

Experimental. The animals employed throughout the experiments were selected from the extensive rat colony of the department of physiological chemistry. These were placed upon their respective predetermined diets at the age of four weeks, being fed previously after weaning upon the so-called adequate growing ration, consisting of 18 per cent alcohol and ether extracted casein, 5 per cent filtered butter fat, 3.7 per cent salt mixture, 5 per cent wheat embryo and 68 per cent dextrin. The A-deficient animals were fed upon similar diet, with the butter fat eliminated and the diet supplemented with an equivalent amount of dextrin. The B-deficient animals were put upon a similar diet, but lacking wheat embryo, with the equivalent amount of dextrin added. Weight and growth records were kept weekly, and the individuals watched closely for developing pathological conditions so characteristic of deficiency diseases. Practically all with B-deficiency disease were taken

for experiments after they had been upon diet between four and five weeks. The A-deficient animals were taken for the most part between the seventh and the tenth week after being put upon diet, but individual differences in susceptibility were marked so that the age factor was not so constant in these groups.

The technical procedure consisted of stretching the animal back down upon an operating board and, in this position after quiet had been attained, body temperature, via rectum, the pulse and respiratory rates were recorded. A rectal injection of a 2.5 per cent solution of chloral hydrate was administered, varying in amounts from 3 to 7 cc. in normal rats, and from 0.5 to 1 cc. in the deficient animals. With this concentration, after a little experience in its administration to different deficient individuals, one was able to administer just sufficient amounts to keep the animals at an even state of anesthesia for sufficient lengths of time to successfully perform the necessary details of operation. After repeated failure to obtain suitable records of pressure from either the carotid or the femoral arteries because of their size, a feasible method was devised which enabled the insertion of a very fine cannula into the abdominal aorta. A slight, longitudinal slit was made in the abdominal muscles, the organs were gently displaced, and the vessel gently clamped off just below the diaphragm. The small glass cannula leading from the specially devised recording manometer and connected with the anti-coagulating reservoir was then carefully tied into the abdominal artery just below the occluding clamp. After suitable adjustments of the apparatus, the clamp was released from the artery and a tracing was obtained. It was not infrequent, due to the nicely adjusted apparatus, that respiration rhythm, as well as the rhythm of heart beat in systolic and diastolic phases, was also recorded (see record 146, fig. 2).

Analysis of data. About seventy-five rats, including both sexes, contribute to the data that follows, about one-half of this number being normals to check equally against the two groups of deficient animals. Although all data were kept in tabular form for the purpose of analysis, it is only necessary here to select typical illustrative cases. Record 150, figure 1, was taken from a normal male rat eleven weeks old, weighing 120 grams, after being on synthetic growing ration. After weaning, this animal was typically active, healthy, with a body temperature of 102°F., and gave a record which showed a maximum pressure of 92 mm. Hg. It is noted that upon removal of the clamp the pressure is immediately effective on the recording manometer, and is maintained at its maximum plateau during the time of the experiment (10 to 15 minutes). The average maximum blood pressure for all (30 individuals) normals from our tabulation is 84 mm. Hg. With the exception of one extremely high, and one extremely low figure, the majority of these did not show conspicuous differences from the computed average.

Among the typical records from the A-deficient animals, we choose pressure curve 146, shown in figure 2. This was taken from a male animal eleven weeks of age, put upon a deficient diet at the age of four weeks; it being on the diet about seven weeks. At the time of the experiment it weighed 47 grams, and showed the usual pathological symptoms. It maintained a rectal temperature of 100.2°F., and exhibited fair activity for animals in its class. The record indicates a maximum pressure of about 70 mm. Hg, and is one of the best we have obtained to show rhythmical pulsations of the heart together with the respiratory undulations. It is conspicuously noted that in all A-defi-

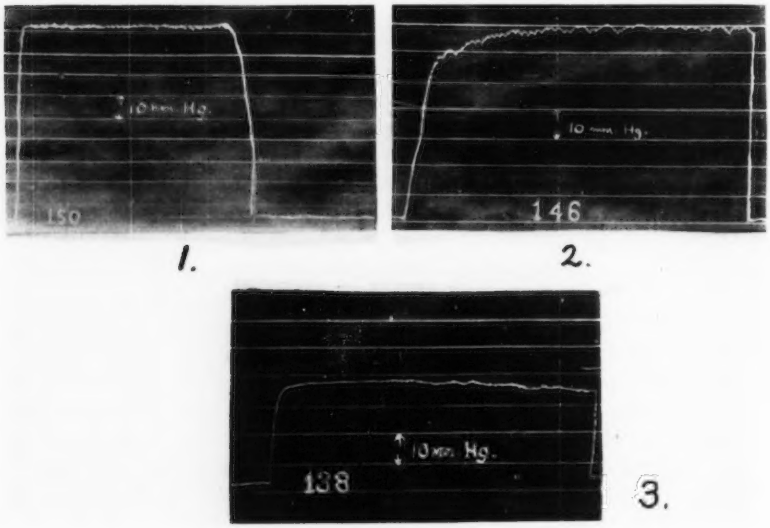

Fig. 1. A typical blood pressure record of a normal rat on a growing ration.
Fig. 2. Typical blood pressure record taken from an A-deficient rat.
Fig. 3. Typical blood pressure record taken from a B-deficient rat.

cient records a sudden rise is followed by a gradual increase, before the full plateau is reached. Records from nineteen animals contribute to the average maximum blood pressure in this group which was found to be 75 mm. Hg. With three exceptional extremes, here as in the normals, the majority of individuals exhibited pressures relatively close to the average.

Record 138, shown in figure 3, is a typical blood pressure curve of female rat nine weeks old, after being upon a B-deficient diet for five weeks. At the time of the experiment this animal weighed 70 grams

and maintained a subnormal temperature of 98°F. The maximum blood pressure was about 40 mm. Hg. The average of sixteen individuals on B-deficient diet was 34 mm. Hg; only five individuals standing sufficiently above the average to approach the average of the normals, the highest of these being 5 mm. below the maximum average. The majority of cases showed maximum pressures in close proximity to the general average. The lowest pressure in any animal was 12 mm. Hg, taken in a very weak individual, weighing only 60 grams, with extreme pathological symptoms and low vitality.

It appears from our experimental data cited that the average normal pressure in the rat is 84 mm. Hg. This closely approximates the figures which Uhlmann found to obtain in the rabbit. In comparison to the normals, the A-deficient animals with few exceptions show a tendency toward a slight but significant sub-normal pressure, the average in these being 75 mm. Hg. In B-deficient animals this maintenance of sub-normal pressure is marked, the average in this group being 34 mm. Hg. The causes contributing to these marked differences in the two deficient groups from each other and from the normals, have not as yet been completely analyzed, but the histological studies now in progress and which we hope to report upon in detail subsequently, give us reason to believe that one of the chief factors is the alteration of the heart muscles.

SUMMARY

1. By observing proper technique it is possible to obtain by a special mercury manometer method, direct blood pressure curves from the abdominal aorta in experimental rats.

2. A convenient method of handling rats during experimentation is outlined and proper lethal doses of chloral hydrate, as an anesthetic, via rectal injections, are indicated; these doses vary from 0.5 to 7 cc. of 2.5 per cent solution, according to size and condition of the rat.

3. Percentage components of the diets used are tabulated.

4. Typical blood pressure curves are shown, and these in connection with other data when analyzed indicate that healthy rats maintain blood pressure fairly comparable to that maintained by rabbits, the average of all normals by this method being 84 mm. Hg. A-deficient animals show slight though significant sub-normal pressure, nineteen animals giving an average maximum of 75 mm. Hg. In the B-deficient group of sixteen animals, the sub-normal pressure was even more marked, the average maximum in this group being 34 mm. Hg.

BIBLIOGRAPHY

(1) Uhlmann: Z. Biol., 1918, lxviii, 419, 457. See tables p. 476–780.
(2) McCarrison: Indian Journ. Med. Research, 1919, 275.
(2b) McCarrison: Studies in deficiency diseases. Oxford Med. Pub., 1921, xx.
(3) Cowgill: This Journal, 1921, lvi, 420.
(4) McCollum and Davis: Journ. Biol. Chem., 1915, xxi, 179
(5) Osborn and Mendel: Journ. Biol. Chem., 1918, xxxiv, 17.
(6) Osborn and Mendel: Journ. Biol. Chem., 1921, xlv, 277.
(7) Sherman and Smith: The vitamines. 1922. See pp. 175–79 and 200–201.

SPONTANEOUS RHYTHMICAL CONTRACTION OF THE MUSCLES OF THE BRONCHIAL TUBES AND AIR SACS OF THE CHICK EMBRYO

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While studying the growth of the lung of the chick embryo in tissue cultures it was found that the muscles of the respiratory tubes exhibit spontaneous rhythmical contraction somewhat resembling that of the muscles of the chick amnion and intestine. This behavior of the embryonic pulmonary muscles is interesting in view of the discussion as to whether the adult bronchioles are able to undergo rhythmical contraction (2), (1), (8). Jackson (3), (4) found that the bronchi of man normally undergo various movements, dilating, contracting, elongating and shortening in rapid succession, and also that rhythmic contractions of the bronchi are evident during respiration. McDowall (7) devised a method by which he claimed it was possible to record changes in the calibre of the bronchioles by using the chest cavity as a plethysmograph.

Bronchial tubes. Rhythmical contraction of the bronchial muscles was not exhibited in cultures of young chick embryos, but began after the ninth day of incubation and continued up to the sixteenth or eighteenth day. It became increasingly difficult to demonstrate beyond this stage although rhythmic contraction was occasionally observed, even in the lungs of chicks that had been hatched for a day.

When the embryo was kept warm and the lung excised into a dish of warm solution (85 cc. Locke solution + 15 cc. chicken bouillon + 0.5 per cent dextrose) and then cut up into the thin small pieces necessary for tissue cultures, the muscles were undergoing rhythmical contraction as soon as observed in the warm box. If, however, the tissue was chilled, a certain period of time elapsed after the preparation was placed in the warm box before rhythmical contraction began. The latent period depended upon the age of the chick and varied from two to fifteen minutes The muscles from embryos older than fourteen days' incubation often became contracted, due to the chilling, and failed to relax, remaining contracted until rigor set in. When rhythmical contraction did not take place at once in cultures from embryos of ten to fourteen days' incubation, it could frequently be initiated by warming the preparation

for a few seconds over an electric-light bulb, but with tissue from older embryos this procedure often brought about an increase in the contraction of the muscles resulting in rigor.

All pieces of the lung exhibited more rhythmical contraction and at a more regular rate after they had grown for twenty-four hours in tissue cultures than immediately after explantation. The pieces of older embryos (eighteen days' incubation) do not grow in tissue cultures and these usually ceased to exhibit rhythmical contraction within ten or fifteen minutes.

The bronchial tubes of embryos between nine and eleven days' incubation[1] were usually straight, with thin, more or less even walls and large lumina. They were encircled here and there by bands of smooth muscle arranged in a single layer just outside of the epithelial lining. There were also a few bundles of smooth muscle passing along the length of the tube. The contraction of these muscles narrowed the lumen and to some extent shortened the tube, resulting in a wavy outline. The contraction sometimes closed the tube throughout its length; in other instances it passed as a wave over the length of the tube, and in still others the tube remained open while the different muscle bundles exhibited feeble and independent contractions and relaxations, too weak or too disorganized to produce any effect upon the tube as a whole. Frequently such feeble contractions took place between more regular and more violent rhythmical contractions which closed the tube about once a minute.

In the lungs of embryos after eleven days' incubation[2] the bronchial tubes were no longer straight, with even walls, but were pitted over their inner surfaces with hollow buds of epithelium growing out into the reticular tissue. These diverticula also contained smooth muscle and when cultures of lungs from embryos of twelve to sixteen days' incubation were kept under observation at 40°C. these muscles were never quiet. Some of them were always contracting or relaxing, resulting in a writhing of the inner surface of the tube, accompanied by less frequent (about once every thirty to sixty seconds) rhythmical closing and opening, also shortening and lengthening, of the entire tube.

The type of muscular contraction exhibited by these smooth muscles was that characteristic for smooth muscle in tissue culture (5), i.e., a slow swinging movement, resulting in a shortening with coincident thickening, in some instances puckering, of the fiber, followed by a slow swaying and elongation, then a period of rest. The contraction of the muscles moved the blood back and forth in the blood spaces along the bronchial

[1] One hundred fifty cultures prepared from ten different embryos were observed.

[2] One hundred eighty cultures prepared from fourteen different embryos were observed.

tubes. Occasionally there were some loose cells and debris within the lumen of the tube, and this material was driven back and forth with each contraction and relaxation. After explantation, where the tubes had not yet healed over, they discharged fluid and debris with each contraction, and aspirated fluid and debris with each relaxation.

In 24-hour cultures the tubes were practically always closed by a new growth of epithelium, while the reticular tissue, smooth muscle, pleural membrane, etc., extended some distance beyond them. In a few instances the epithelium, instead of simply closing in the cut end, grew out in the form of a membrane, composed frequently of ciliated cells, whose cilia extended down into the hanging drop, and beat violently and incessantly.

The rate of the rhythmical contraction was usually about once in thirty seconds, but it could be largely controlled by the temperature at which the preparation was observed. For instance, a tubule closing and shortening throughout its length once in every ten or fifteen seconds at 40°C., closed once every thirty seconds at 38°C., once every minute at 37°C., and when further cooled exhibited only feeble contraction and relaxation of the muscle bands without closing the tube, while at 36°C. all muscular movement had ceased. The tubules in the same piece did not always contract at the same rate, and two lying side by side frequently contracted at markedly different rates unless the culture was kept quite warm, 40°C. to 41°C. This temperature frequently brought about such violent rhythmical contraction that the piece became torn loose from the coverslip and the new growth drew in around the explanted piece. For this reason observations were usually carried on at about 38°C.

The bronchial tubes continued to exhibit rhythmical contraction in the tissue cultures for many days, in some cultures for two weeks. A bronchiole in a culture from the lung of a ten-day chick embryo, followed from day to day, continued to undergo rhythmical contraction at about the same rate for ten days.

Air sacs. The avian lung exhibits such a special architecture, i.e., air sacs, recurrent bronchi and labyrinthic connections between air passages[3] that it is questionable whether it can be compared with the mammalian lung. Especially is this true in regard to the air sacs, as the lungs of birds have no terminal alveoli containing residual air; instead each lung has five greatly enlarged sacs extending outside of the pleural cavity and from which recurrent bronchi form a system of communicating tubes through which the air flows.

These sacs are lined with endoderm and covered by a loose reticulum of smooth muscle fibers. When the air sacs were studied in tissue cultures

[3] For a description of the morphology and embryology of the avian lung the reader is referred to the publications of Locy and Larsell (6).

it was found that the muscles were undergoing movement as soon as explanted and continued to do so for many days. Each muscle bundle exhibited contraction and relaxation, but seldom at the same time or at the same rate as its neighbors, so that the whole surface of the piece was continually writhing without any definite, coördinated contraction of the whole piece.

Nerve fibers occasionally grew in these cultures of the lungs, but in such a small proportion (5 per cent) of the cultures that it seems evident that in the chick embryo of ten to sixteen days' incubation the spontaneous rhythmical contraction is a function of the muscle itself.

BIBLIOGRAPHY

(1) Dixon and Brodie: Journ. Physiol., 1903, xxix, 97.
(2) Einthoven: Pflüger's Arch., 1892, li, 367.
(3) Jackson: Bronchoscopy and esophagoscopy. Philadelphia, 1922.
(4) Jackson: Amer. Journ. Med. Sci., 1923, cixv, 313.
(5) Lewis: Contributions to Embryology, 1920, ix, 191; Carnegie Inst. Wash. Pub. 272.
(6) Locy and Larsell: Amer. Journ. Anat., 1916, xix, 447; xx, 1.
(7) McDowall: Journ. Physiol., 1923, lvii, lv.
(8) Trendelenburg: Arch. f. Exper. Path. u. Pharm., 1912, lxix, 77.

THE EFFECT OF PROLONGED FASTING FOLLOWED BY REALIMENTATION ON GASTRIC SECRETION

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Previous experiments seem to indicate that there is an improvement in general nutritional conditions of the body as a result of prolonged fasting (1). Also, that there is a temporary increase in the basal metabolic rate after the fast has ended and the dietary of the subject is again comparable to that of the pre-fasting period. This experiment was carried out with the idea of determining whether significant changes occur in the amount and acidity of the gastric juice during realimentation after prolonged fasting.

The literature on the factors which influence gastric secretion, together with the historical setting and the development of experimental methods, has recently been reviewed by Carlson (2). References in this article are made only to experiments which have direct bearing on the problem under discussion.

EXPERIMENTAL METHOD. Two Pavlov pouch dogs were used in this research. Operations converting a portion of each stomach into a pouch were made two months (May 21, 1923) before taking experimental data. This amount of time was allowed to pass between the operation and the recording of data in order to assure general systemic recovery of the dogs, and a normal condition of the secretory mechanism of the pouch. Beginning with the fifth week (June 28, 1923) after the operation the dogs were fed a daily maintenance diet which remained constant for the pre- and post-fasting periods. This precaution is necessary to prevent quantitative changes in the gastric secretion resulting from quantitative or qualitative changes in the food given. The dietary of each dog is given in table 1. The ingredients of each diet were weighed, thoroughly mixed, and each animal fed the maintenance amount at one daily feeding. About once a week small amounts of bones were added to the diets of each dog. It was found that the gnawing of the bones, or the changes in the mineral metabolism due to the bones eaten, had a marked effect on the gastric secretion during the day that the bones were fed. Whether this stimulation lasts

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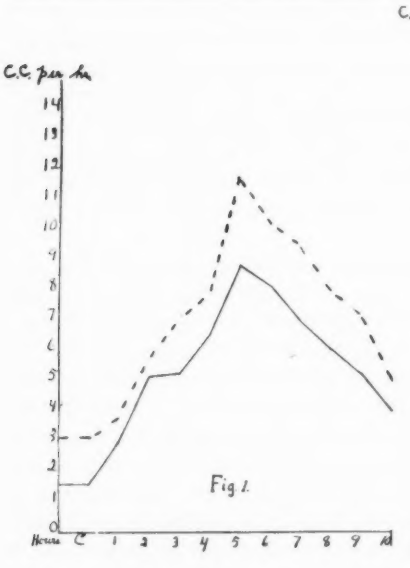
longer than a day has not yet been determined. However, we believe that this diet is not adequate without the occasional addition of a small amount of bones. The addition of bones becomes especially necessary after fasting. During the fasting there seems to be a depletion of calcium and other minerals. The small amount of minerals present in the diet of bread, meat and milk does not seem to be quite sufficient to restore the minerals lost during fasting and maintain a normal daily balance.

TABLE 1

Daily diets of the dogs during the pre- and post-fasting periods

SUBJECT	HASHED LEAN MEAT	BREAD	WHOLE MILK
	grams	*grams*	*cc.*
Dog I	300	100	150
Dog II	200	100	150



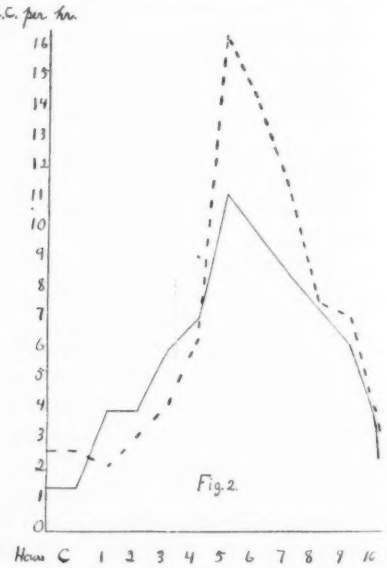


Fig. 1. Dog I. Gastric secretion before and after fasting. The unbroken line shows the average hourly secretion during the pre-fasting period (22 days). The average hourly secretion during the post-fasting period (44 days) is indicated by the broken line. *C* indicates the average number of cubic centimeters of gastric juice secreted during each period one hour before feeding.

Fig. 2. Dog II. The unbroken line indicates the average hourly secretion before fasting (22 days), the broken line after fasting (44 days). *C* indicates the average number of cubic centimeters of gastric juice secreted during each period, one hour before feeding.

The control or pre-fasting period was twenty-two days in duration (July 21, 1923 to August 11, 1923). The average body weight of dog I was 17.2 kilos. The greatest deviation from this average was 0.12 kilo. The average body weight for dog II was 12 kilos. The greatest deviation from this was 0.05 kilo.

The fasting period (August 11 to September 7, 1923). During the twenty-seven days of fasting no food was given the dogs. They were allowed water ad libitum, excepting the twenty-third and twenty-sixth fasting days. On these days special experiments on the effect of large amounts of water on the gastric secretion during prolonged fasting controlled the water intake of the animals as will be described later. The body weight of dog I was reduced from 17.4 kilos to 12.1 kilos, a loss of 34.5 per cent of the control body weight. The weight of dog II was reduced from 12.1 kilos to 7.9 kilos, a loss of 34.5 per cent of the control weight. No untoward symptoms other than a loss in body weight were noticed in either dog during the fasting.

The post-fasting or period of realimentation (September 7–October 20, 1923) 44 days. During this time the food, water and collection of gastric juice was as nearly identical to that of the control period as could be maintained.

COLLECTION OF GASTRIC JUICE. Two months after the operation and five weeks after the daily dietary of the dogs consisted of the maintenance requirements, pure gastric juice was collected from the pouches during the pre- and post-fasting periods, according to the following routine:

1. The Pavlov pouches were dressed and the continuous secretion collected for one hour.

2. The animals were fed the maintenance diet and hourly collections of juice made over a period of 8 to 10 hours.

3. Water ad libitum was given the animals at certain intervals, i.e., at the end of the fourth hour after feeding.

Practically all of the gastric juice secreted during the entire fasting period was collected. This was made possible: *a*, because of the small amount of juice secreted, and *b* the collecting devices were securely placed at night and the dogs muzzled to prevent them tearing off the bandages or stimulating psychic secretion by licking the pouches.

RESULTS OF THE EXPERIMENT. The average hourly secretion of gastric juice for the pre- and post-fasting periods is shown in figures 1 and 2. The number of cubic centimeters plotted in the first space of the pre- and post-fasting curves, under *C*, indicates the average continuous secretion for the hour before the dogs were fed. The dogs were fed at the beginning of the hour marked *1* and hourly collections of gastric juice recorded up to the end of the 10th hour. The average number of cubic centimeters of juice secreted for each hour during each period is given in table 2.

The fasting secretion, as has already been stated, was collected practically over the entire fasting period. Many of the collections were made over single hours but during a part of each day and during the night the average secretion over a period of several hours was determined. This average hourly secretion during the 27 fasting days, for dog I amounted to 1.2 cc.; for dog II, 1.1 cc.

DISCUSSION OF RESULTS. From the curves, figures 1 and 2, it can be readily seen that the gastric secretion of dogs during realimentation after prolonged fasting is markedly influenced by fasting. This influence is apparent at three different phases of the daily secretion curve; *1*, both dogs show an increase in the continuous secretion, i.e., the amount of juice secreted on an empty stomach. This is not as marked in dog II as in dog I. *2*, In case of both dogs the amount of gastric juice secreted the first three hours after feeding, i.e., the appetite juice, is relatively less during the post-fasting period than during the pre-fasting period. This seems to indicate that the psychic mechanism due to seeing and eating food is not stimulated nearly so markedly by the same amount of food prepared

TABLE 2

Tabulation of average number of cubic centimeters of gastric juice secreted per hour for the pre- and post-fasting periods

SUBJECT	PERIOD	CONTROL	1 HR.	2 HR.	3 HR.	4 HR.	5 HR.	6 HR.	7 HR.	8 HR.	9 HR.	10 HR.
Dog I.....	Pre-fasting	1.6	3.0	5.1	5.2	6.5	8.8	8.2	7.0	6.1	5.2	4.0
Dog I.....	Post-fasting	3.1	3.8	5.8	7.1	8.0	11.8	10.2	9.5	8.0	7.2	5.0
Dog II.....	Pre-fasting	1.5	4.0	4.0	6.0	7.1	11.2	10.0	8.6	7.4	6.1	2.4
Dog II.....	Post-fasting	2.6	2.2	3.2	4.2	6.4	16.4	14.4	11.4	7.6	7.2	3.2

in the same way during intense hunger as it is under normal conditions. The food given each dog before and after fasting was each day carefully weighed and mixed to a soft pulp. Before fasting the dogs always displayed considerable appetite at the feeding period, but the length of time taken to consume the food was noticeably longer than after fasting, and in most cases there was an increase in the amount of juice secreted during the next hour. After fasting this was markedly changed. The dogs voraciously devoured their food in just a moment, always looking about for more and giving other signs of incomplete satiety. The gastric juice on these occasions was not increased during the next hour after eating. In fact, in many instances it was markedly lessened. Just what the mechanism on the psychic secretion is that is responsible for this inhibition is difficult to state. It may be due to a lessened amount of chewing or to tasting for a shorter period of time, or it may be that intense hunger indicates that the chemical stimulation of food substances has not acted on the gastric glands for a long period of time thereby lowering the tonicity of the glands.

We believe that psychic influences are far less potent in stimulating gastric glands in a state of lowered tonicity than they are if the tonicity of the glands is high. Further investigation along this line is necessary before definite conclusions can be drawn.

3. The influence of water. The stimulating influence of water upon the gastric secretion, under various experimental conditions has been described by Pavlov, (3) Bergleim et al (4), Carlson (5), Ivy (6), Southland (7) and others. In order to keep the stimulating influence of water during the pre- and post-fasting periods as nearly identical as possible, a large pan of water was given the dogs at the end of the fourth hour after feeding. This water remained in the cages during the rest of the day and the dogs were allowed to drink ad libitum. At night the water was removed and no more given until the end of the fourth hour after feeding the next day. This method of administering the water brought its stimulating influence in at a phase of digestion when the heightened activity of the gastric glands is at its maximal. Under these conditions the water calls forth a response much greater than that which was produced during the four previous hours after food. The amount of gastric juice secreted from the 4th to 9th hour after food and 1 to 4 hours after water was much greater after fasting than before. The stimulating effect of water was usually noticed at the end of the first hour, always at the end of the second hour, and continuing for 4 to 6 hours.

Ivy explains the increase in gastric secretion which occurs after fasting as due to the stimulating effect of the HCl on the duodenal mucosa. Ivy suggests that the duodenum has been free from acid, although not complete, and food content for a long period of time, and hence the duodenum and jejunum are in a comparable condition to that of a Thiery's fistula of the duodenum and jejunum. We believe that another important factor, contributing to increase the gastric secretion after fasting, may be found in a changed condition of the gland cells. Numerous experiments on realimentation, which we have recently observed, indicate clearly that immediately after breaking a fast of five days or more, there is a temporary accumulation of fluids in the tissue, due to the hydration of fasting. This condition seems only to be present during the first few days of realimentation, after which it seems that the excess of fluid is replaced by active tissue. It is quite possible, however, that the permeability of the cell membranes is influenced for a long period after fasting, thereby allowing a greater exchange of fluid substances between secreting cells and capillaries and lymph spaces. At the end of the fourth hour after food, the gastric glands are actively secreting and in a high state of tonus. A temporary condition of hydremic plethora due to the ingestion of water together with increased permeability acting on the glands in heightened tonus would result in an increase in functional activity of the cells. It should be noted

in this connection that the marked increase in gastric secretion was not apparent immediately after breaking the fast, but came several days later. This delay may in part be due to an actual recuperation of the gastric glands and in part to the general retention of fluids by the body which has already been mentioned. Four to five weeks after fasting the gastric juice secreted again approached the pre-fasting amount and the hourly collections were discontinued November 20, 1923. Further studies on these dogs showed that water given 8 to 24 hours after eating had little effect on the gastric secretion unless accompanied by marked thirst. During the fasting period water was always present in the dogs' cages excepting on the twenty-third and twenty-sixth fasting days. On the twenty-third fasting day water was withheld for eight hours in an attempt to create a thirst. At the end of eight hours water was given each dog. During the two hours before drinking water, the fasting secretion of dog I averaged $\frac{1}{2}$ cc. per hour. After the administration of water it averaged $1\frac{3}{4}$ cc. per hour for 2 hours, then returned to the former level. The total acidity after the ingestion of water increased from 0.065 per cent to 0.1185 per cent. Similar results were obtained with dog II. On the twenty-third fasting day it was also deprived of water for eight hours. The starvation secretion for the two hours previous to water drinking averaged $\frac{1}{2}$ cc. per hour. After water it increased to 1 cc. per hour for two hours. The total acidity increased from 0.06 per cent before water to 0.09 per cent after water. The amount of increase in gastric juice was slight but appreciable in both cases. On the twenty-sixth day of fasting water was withheld from both dogs for $12\frac{1}{2}$ hours, after which 600 cc. were administered by means of a stomach tube to dog I (the larger of the two dogs) and 400 cc. to dog II, with the following results: 1 to 3 hours previous to the administration of water, the gastric secretion of dog I averaged $\frac{1}{2}$ cc. per hour. Total acidity, 0.00 per cent. After the administration of water it increased to $1\frac{5}{6}$ cc. for two hours with a total acidity of 0.091 per cent. Dog II secreted 1 cc. per hour for the four hours previous to the administration of H_2O. Total acidity 0.091. After introducing 400 cc. H_2O, with a stomach tube, the amount secreted increased to only $1\frac{3}{4}$ cc. per hour but the total acidity increased to 0.2188 per cent. Pavlov (3) states that water must be accepted as a chemical excitant of gastric secretion although only a weak one. It seems from these experiments that the effectiveness of water as a gastric secretory stimulant depends entirely upon the state of tonicity of the secreting glands. If given during the height of digestion the amount of juice secreted for the next 3 to 4 hours may be increased two or three fold, whereas if much larger quantities are given during prolonged fasting (23rd to 26th fast day) the increase is very slight.

The effect of fasting on the acidity of the gastric juice. No marked changes occurred in the acidity of the gastric juice after fasting. However, the

greatest amount of acidity occurred during the post-fasting period, but this was always coincident with the marked increase in the amount of juice secreted per hour. The maximal total acidity, 0.51 per cent, was reached at a time when the Pavlov pouch secreted 20.3 cc. per hour. This, although high, is within normal limits.

Secretion of gastric juice during fasting. In these dogs the hourly secretion of gastric juice, during the twenty-seven days of fasting, at no time exceeded the hourly secretion of gastric juice of the empty stomach during the control period or the after fasting period. In these experiments we could not confirm the findings of Boldyreffs (8), i.e., after 3 to 4 days of fasting, the gastric secretion becomes abundant periodically, and later becomes so abundant as to inhibit the motility of the fasting stomach. Carlson (9) found no increase in the continuous secretion of man fasting five, eight and fifteen days. But there was an augmentation in the continuous secretion of the subject after breaking the fast. I would like to emphasize the fact that in this experiment the utmost care was taken to prevent the dogs from licking the pouches or eating bits of the dressing. This can be accomplished only in most cases by muzzling the animals when they are not under direct vigilance. At no time during the fasting period were we able to stimulate psychic secretion by having the animals see or smell food. This is another example of the inhibitory effect of intense hunger on the psychic mechanism.

After the dogs had regained their initial body weights on the pre-fasting maintenance diet, dog I was used for other experimental purposes. The diet of dog II was reduced on November 12, nine weeks after fasting, from 200 grams hashed raw lean meat, 100 grams bread, 150 cc. whole milk, to 175 grams meat, 75 grams bread, 150 cc. whole milk. The weight of the dog November 12, 1923, was 12 kilos. On December 1, 1923, the animal weighed 12.15 kilos. This agrees with previous findings on realimentation after fasting; i.e., the organism seems able to utilize food on a more economical basis indicating an improvement in the general nutrition condition.

SUMMARY

1. Intense hunger seems to exert an inhibitory influence on the psychic secretions.

2. There is no increase in the continuous gastric secretion of dogs during twenty-seven days of fasting. The gastric secretion during twenty-seven days of fasting at no time increased in amount or acidity above the secretion of the empty stomach under normal conditions.

3. There is a slight increase in the continuous secretion during realimentation after fasting, but a marked increase in the secretion following feeding. This may persist for four to six weeks after breaking the fast.

I wish to thank Professor Carlson for helpful criticisms and assistance during the progress of this work.

BIBLIOGRAPHY

(1) KUNDE: Journ. Metabol. Research, 1923, iii, 399.
(2) CARLSON: Physiol. Review, 1923, iii, 1.
(3) PAVLOV: The work of the digestive glands. London, 1910.
(4) BERGEIM ET AL: Journ. Biol. Chem., 1914, xix, 345.
(5) CARLSON ET AL: This Journal, 1914, xxxiii, 91.
(6) IVY: This Journal, 1918, xlvi, 420.
(7) SOUTHLAND: This Journal, 1921, lv, 258.
(8) BOLDYREFF: Arch. d. Sci. Biol., 1905, xi, 1.
(9) CARLSON: This Journal, 1918, xlv, 120.

BIOLOGICAL FOOD TESTS

VI. Further Experiments upon the Vitamin A and B Content of Citrus Fruit Products

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In a previous report (1) certain data concerning the vitamin A content of some citrus fruit products were presented. It was shown that orange oil extracted by ether or by pressure, orange peel and orange juice are relatively rich in vitamin A; that lemon peel is less valuable in this respect; that cold pressed lemon oil and dried grapefruit peel are ineffective. In continuation of these studies, information has further been collected concerning the vitamin A content of the ether extracts of lemon peel and of grapefruit peel, of cold pressed grapefruit oil, of lemon juice and of fresh and concentrated grapefruit juice. In addition both vitamin A and vitamin B contents of several cooked orange peel confections have been determined. This latter test was undertaken in the hope of discovering some means of utilizing for human consumption the marked vitamin A value of orange peel. Some experiments upon the use of fresh dried ground pulp as an ingredient of dairy cow feed have recently been carried on by California orange growers with what are reported to be excellent results. It was thought worth while to test the possibility of retaining a certain proportion of the suprisingly large vitamin A content of orange peel in those few preparations in which the peel is utilized. Accordingly two types of crystallized orange peel, commerical and home-made, and two similar preparations of orange marmalade were fed as sole sources of vitamin A and in another series as sole sources of vitamin B.

Methods. The method of testing was that described in a previous paper (1). The animals used were rats from our own colony, and the basal diet our usual casein-dextrin-crisco-salt mixture. Butter fat was used as source of vitamin A in the vitamin B tests and for the control animals, and dried brewery yeast was similarly employed as the source of vitamin B in all vitamin A tests and for the control animals. The preparation under investigation was given in all cases separately from the basal diet, in weighed daily doses. Accurate records of food intake were made, weights taken twice a week, and autopsies performed upon all animals which died during the experiments. Only the curative method of testing was used

in this series of experiments. In all cases the animals were placed at weaning time upon the basal diet with butterfat or yeast additions according as vitamin A or vitamin B was sought. In twenty-five to thirty days xerophthalmia developed in all cases of vitamin A deficiency, and emaciation and partial paralysis or leg weakness in cases of vitamin B deficiency. The citrus product under investigation was then added to the diet, and if growth resulted the feeding was continued for twenty to twenty-four weeks.

TABLE 1

Curative tests for vitamin A in grapefruit and lemons

NUMBER OF RATS USED	DURATION OF TEST	PREPARATION TESTED	MAXIMUM AMOUNT USED	RESULTS
	weeks			
5	5 to 9	Concentrated grapefruit juice	15 to 30 cc.	Xerophthalmia, declining weight and death
6	9 to 14	Fresh grapefruit juice	15 to 30 cc.	Xerophthalmia and declining weight somewhat relieved, death postponed 4 or 5 weeks longer than occurred with the concentrated juice
4	6 to 8	Grapefruit peel oil (pressed)	0.2 to 1 gram	No protection
2	4 and 6	Ether extract of grapefruit peel	0.2 to 1 gram	No protection
4	5 to 14	Lemon juice	15 to 30 cc.	With one exception decline and death postponed 4 to 13 weeks
4	9 to 24	Ether extract of lemon peel	0.2 to 1 gram	Three of the animals were able to make slight gains for 19, 21, or 24 weeks. The eye condition was much alleviated in all but one case

Vitamin A in grapefruit juice. Two kinds of grapefruit juice were tested, a concentrated bottled juice put up by a citrus by-products factory in southern California, and the fresh juice prepared daily from specimens of the fruit purchased in the retail market. The concentrated juice was a viscid brown syrup prepared by the usual method of boiling under reduced pressure. With neither juice was any curative effect upon the eye condition noted, and in all cases death was postponed only a short time. A slight but definite advantage could be observed from the use of the fresh instead of the bottled juice since the animals lived nine to four-

teen weeks on the former and only five to nine weeks on the latter. These results are shown in chart 1 and table 1.

Vitamin A in grapefruit oil and in ether extract of grapefruit peel. An oil obtained by pressure from grapefruit peel[1] was fed in 1-gram doses to four young rats suffering from acute vitamin A deficiency, without the slightest alteration of their symptoms. An extract of dried grapefruit peel[2] was made by means of U. S. P. ether, and tested by feeding to two young rats. The same negative results were noted as with the pressed grapefruit oil. These results are shown also in chart 1 and table 1.

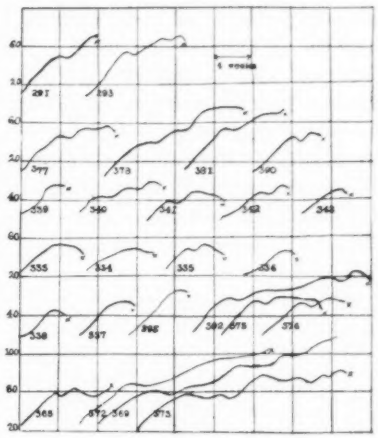

Chart 1. Growth curves of rats fed lemon and grapefruit products as source of vitamin A.

Basal diet and 0.3 gram of dried brewery yeast given in each case in addition to source of vitamin A, administration of which began at end of first four weeks.

Rats 365, 372, 369, 373 fed 0.2 to 1 gram ether extract of lemon peel. Rats 392, 395, 375, 376, fed 15 to 30 cc. fresh lemon juice. Rats 338 and 337 fed 0.2 to 1 gram ether extract of grapefruit peel. Rats 333, 334, 335, 336, fed 0.2 to 1 gram pressed grapefruit oil. Rats 339, 340, 341, 342, 343 fed 15 to 30 cc. reconstituted concentrated grapefruit juice. Rats 377, 378, 381, 390, 291, 293 fed 15 to 30 cc. fresh grapefruit juice.

Vitamin A in lemon juice. Fresh lemon juice was fed to four rats in portions similar to those used with the grapefruit juice. Some difficulty was encountered in inducing the animals to take this juice although both orange and grapefruit juice were readily consumed. The acid of the lemon juice was neutralized with sodium bicarbonate, small amounts of sugar were added, and all other liquid removed, but in nearly all cases, stubborn refusal to partake continued. It is felt, therefore, that the negative results obtained cannot be ascribed confidently to the nature of the juice. The deaths of the animals from vitamin A deficiency were in any case postponed several weeks. See chart 1 and table 1.

[1] This oil was supplied through the kindness of Mr. W. J. Bush of the W. J. Bush Citrus Products Company, of National City, California, who described the process by which it was made thus: "Our grapefruit oil is obtained by a mechanical method of expression, which causes the oil cells to spray into a descending stream of juice from which the oil is separated by means of high-speed centrifugal separators."

[2] The dried grapefruit peel used in this experiment as well as the dried orange peel and lemon peel used in all our vitamin studies was supplied us through the kindness of Mr. C. P. Wilson and the Research Laboratory of the California Fruit Growers Exchange. These products were dried at 65 to 70°C. and in all cases averaged 34 per cent of the fresh peel and 8 to 10 per cent of the whole fresh fruit.

Vitamin A in the ether extract of lemon peel. An extract of dried lemon-peel made with U. S. P. ether was used in the case of four rats suffering from xerophthalmia and other conditions characteristic of vitamin A deficiency. Three of these animals made slow gains in weight for from nineteen to twenty-four weeks, and exhibited practically normal eye condition. The dosage was at first equivalent to that used in the experiments on the similar ether extracts of orange peel and grapefruit peel, but was increased gradually from two-tenths of a gram to nearly one gram daily. This relative inadequacy of the ether extract is comparable with the similar inadequacy exhibited by the dried lemon peel itself and the total failure of pressed lemon oil recorded in the earlier report on vitamin A in citrus fruits (1). See chart 1 and table 1.

TABLE 2

Summary of vitamin A content of citrus fruits

	ORANGE	LEMON	GRAPEFRUIT
Pressed oil of peel	0.2 gram sufficient for protection and full growth	No protection	No protection
Ether extract of peel	0.1 gram sufficient for protection and full growth	1 gram doses provided for slow growth	No protection
Dried outer peel	0.5 to 1.0 gram sufficient for full growth	1 gram doses provided for slow growth	No protection
Juice	12 to 30 cc. sufficient for full growth	15 to 30 cc. insufficient to postpone decline and death longer than 4 to 13 weeks	15 to 30 cc. insufficient to postpone decline and death longer than 9 to 14 weeks

Comparison of orange products with lemon and grapefruit products as sources of the vitamins. The striking contrast afforded by the value of orange juice, orange peel and orange oil as sources of vitamin A as compared with lemon and grapefruit juice, peel and oil used for the same purpose. is worthy of attention. These fruits are structurally and chemically so closely related that this divergence in vitamin A content is unexpected. Similar differences in vitamin C and vitamin B content have not yet been reported, and indeed, evidence of parallel values among the citrus fruits as to the vitamin B has been brought forward by Osborne and Mendel (2). These investigators reported excellent growth upon 10 cc. daily doses of orange, lemon and grapefruit juices, and summarize the value of these juices in this regard as similar to that of milk. In the same communication

a preliminary statement as to vitamin A in citrus fruits is in entire agreement with our findings. A later preliminary article by the same authors (3) confirms this statement concerning the presence of vitamin A in orange juice, although no such confirmation of the supposed negative value of lemons and grapefruit is offered.

Comparisons of the anti-scorbutic action of oranges, lemons and limes have frequently been made, but no similar figures for grapefruit are available. Davey (4) reported the minimal dosage for the protection of guinea pigs against scurvy as 1.5 cc. of lemon juice, 1.5 cc. of orange juice and 5 cc. of lime juice. Chick, Hume and Skelton (5) found lemon juice to be about four times as potent as lime juice. It is probable that there exists no appreciable difference in vitamin C content of oranges and lemons. Whether this statement may be extended to grapefruit juice as well cannot be established from existing data. Studies of the antiscorbutic action of this juice are in progress in this laboratory.

Relation of lipochromes to vitamin A in citrus fruits. It is interesting to note that in our experiments, as summarized in table 2, incidence of vitamin A parallels depth of pigmentation, as observed in both pulp and peel of the orange as contrasted with the lemon and the grapefruit. A preliminary study of the lipochrome content of these fruits has yielded data as to a probable relation between the character of pigments present and the vitamin A content. These results will be reported soon.

Vitamins A and B in orange marmalade. Two varieties of orange marmalade were tested, a commerical and a homemade product. The former was prepared in the laboratory of the Division of Fruit Products of the College of Agriculture of the University of California under the direction of Mr. W. V. Cruess who kindly furnished us with the samples used. The process used in making this marmalade is described as follows:

1. A mixture of 1 part orange and 1 part lemon sliced in pieces one-fourth inch in thickness is boiled with three parts of water for sixty minutes, pressed and filtered.

2. Orange peel is sliced thin, boiled 20 minutes, drained and 10 per cent by weight then added to the juice obtained in (1).

3. Granulated sugar in the proportion of one pound per pint of juice and peel is then added to the mixture and the whole boiled till a temperature of 220°F. is reached. When the marmalade has cooled to 150°F. it is sealed in jars in vacuo and pasteurized at 175°F. for thirty minutes.

It is plain that the juice and peel used were subjected to high temperatures for considerable periods of time under conditions which would allow a certain amount of oxidation, and consequent destruction of vitamin A.

It was thought worth while to ascertain the extent to which the vitamin A of peel and juice could be conserved in the marmalade by a modification of these conditions. Accordingly the second marmalade was made by a well-known home process which is described as follows:

The juice of two parts orange and one part lemon is extracted and the peel run though the food chopper. To the combined peel and juice three volumes of water are added and the mixture allowed to stand over-night. It is boiled ten minutes, allowed to stand over-night again, and then cooked one hour or until tender. An equal measure of sugar is added and the mixture boiled until it will form a jelly. The temperature reached during the last boiling is usually 204° to 206°F.

TABLE 3

Curative tests for vitamin A in commercial and home-made orange marmalades and crystallized orange peel

NUMBER OF RATS USED	DURATION OF TEST	FOOD TESTED	MAXIMUM AMOUNT USED	RESULTS
	months		*grams*	
4	1	Commercial orange marmalade	3	The animals were practically stationary in weight, 1 died, all developed xerophthalmia
5	4	Home-made orange marmalade	3	The animals made fair gains in weight. All developed xerophthalmia on basal diet which was much alleviated on feeding the marmalade, although the condition did not entirely disappear
4	4	Commercial crystallized orange peel	3	The animals made poor gains in weight. All developed xerophthalmia, which did not clear up
3	4	Home-made crystallized orange peel	3	The animals made good gains in weight when taken off commercial orange marmalade diet and put on home-made crystallized orange peel. The eye condition improved remarkably

The total time of boiling and the temperatures reached are obviously less than the corresponding figures used in the commercial process although quantitative comparison was not attempted.

Vitamin A in orange marmalades. These marmalades were fed to young rats as sole source of vitamin A in addition to the usual basal diet and dry brewery yeast, the latter to provide an ample supply of vitamin B. The largest amount of marmalade which the animals could be induced to consume consistently was three grams daily. Upon this intake of the commercial marmalade all of the rats failed to recover from the symptoms

of vitamin A deficiency. All showed severe xerophthalamias and one died. The animals fed a similar amount of the homemade marmalade, however, made fair increases in weight and regained nearly normal eye conditions. These results are shown in table 3 and chart 2. It appears probable that the process used in making orange and lemon marmalade affects appreciably the vitamin A content of the product. It should

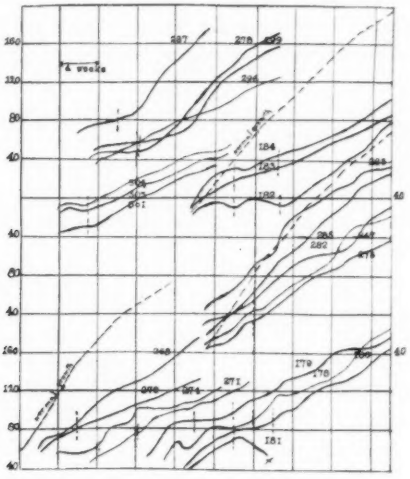

Chart 2. Growth curves of rats fed commercial and home-made orange marmalades and candied orange peels as sole source of vitamins A and B.

Basal diet and 0.3 gram dried brewery yeast were given in each test for vitamin A in addition to citrus product tested.

Basal diet and 10 per cent butterfat were given in each test for vitamin B in addition to citrus product tested. Administration of citrus product was begun at dotted lines.

Rats 268, 270, 274, 271 fed 3 grams commercial candied orange peel as source of vitamin A. Rats 301, 303, 304, fed 3 grams of same as source of vitamin B. Rats 275, 247, 282, 285, 283, fed 3 grams home-made orange marmalade as source of vitamin A. Rats 287, 278, 299, 296, fed 3 grams home-made orange marmalade as source of vitamin B. Rats 178, 179, 180, 181 fed 3 grams commercial orange marmalade as source of vitamin A. At second dotted line 3 grams home-made candied orange peel were substituted for the marmalade. Rats 182, 183, 184, fed 3 grams commercial orange marmalade as source of vitamin B. At second dotted line 3 grams home-made candied orange peel were substituted for the marmalade.

be pointed out, however, that the commercial marmalade used in this comparison contained a somewhat larger proportion of lemon juice and lemon peel to orange (orange to lemon, 1 to 1) than did the home-made preparation (orange to lemon, 2 to 1). In view of the decidedly lower value of the lemon as source of vitamin A, it must be admitted that this

difference in composition may help to explain the differences in the curative action observed.

Vitamin B in orange marmalades. Curative tests made upon young rats which had been reduced to the typical emaciation and paralysis of vitamin B deficiency showed similar but less marked differences between the two marmalades. These animals were given the usual basal diet in which 10 per cent of butter fat had been incorporated to insure a sufficient supply of vitamin A, but were given no source of vitamin B until the marmalade was administered. Doses of 3 grams daily of the commercial

TABLE 4

Curative tests for vitamin B in commercial and home-made orange marmalades and crystallized orange peel

NUMBER OF RATS USED	DURATION OF TEST	FOOD TESTED	MAXIMUM AMOUNT USED	RESULTS
	months		*grams*	
3	1	Commercial orange marmalade	3	Rats stationary in weight, paralyzed position,—bad condition
4	4	Home-made orange marmalade	3	The animals made poorer gains than in vitamin A test, were nervous, had poor coats of fur, but were not paralyzed
3	4	Commercial crystallized orange peel	3	The animals made poor gains, same as for vitamin A test, had rough, poor coats, showed slight paralysis
3	4	Home-made crystallized orange peel	3	The animals made fair gains after the change from commercial marmalade to home-made peel, were not paralyzed, regained good coats of fur

marmalade brought about no improvement. Similar dosage of the homemade marmalade, however, produced a distinct improvement in the condition of the animals, but was not sufficient to stimulate normal growth. These results are illustrated in table 4 and chart 2. The considerably less potent effect of the orange as source of vitamin B than of vitamin A as shown in this experiment is borne out by our later experiences.

Vitamins A and B in candied orange peel. Two specimens of crystallized or candied orange peel were tested, a commercial peel prepared by the California Fruit Confection Company of Camarillo, California, by a

process about which we were not able to gain any information, and a homemade crystallized peel. The latter was prepared as follows:

The orange peel was covered with cold water and boiled till tender. After the peel was cool the inner white skin was removed and the peel sliced. This was then cooked five minutes in a hot syrup of one part water and two parts sugar. The peel was removed from the syrup, excess sugar scraped off, and the remaining substance dried at a low temperature and ground. The excess syrup was removed from the commercial peel, which was dried and ground before it was fed to the animals.

The rats ate their daily 3-gram portions of these candied peels without enthusiasm, and could not be induced to take larger amounts.

Vitamin A in candied orange peel. The animals fed the commercial peel as sole source of vitamin A made small gains in weight, far below the normal, and showed little improvement in the xerophthalmia which had developed on their previous vitamin A free diet. The animals fed the homemade candied peel made good gains in weight, and their eye condition improved remarkably within a few days. These rats had been fed the commercial marmalade described previously and had declined upon it to a marked state of vitamin A deficiency. These results are illustrated in table 3 and chart 2.

Vitamin B in candied orange peel. The same general results were observed when rats were fed these peels as source of vitamin B. All the animals were reduced to a definite condition of vitamin B deficiency characterized by emaciation, rough fur and paralyzed position of the hind legs. They were then given 3-gram daily portions of the peels. No improvement was noted in those fed the commercial product although small gains in weight were recorded. Those given the homemade candied peel made fair gains in weight, were not paralyzed, had good coats of fur, and in general returned to a more nearly normal condition. The value of the peel as well as the marmalade as a source of vitamin B was plainly less than their value as a source of vitamin A. These results are shown in table 4 and chart 2.

These comparisons of the vitamin A content of citrus preparations made more evident the well understood fact that both vitamins A and B are thermolabile under the usual conditions of oxidation and hydrolysis accompanying most cooking processes. That even slight modifications of the time and temperature of cooking may produce appreciable differences in the amount of destruction of these vitamins is, however, worthy of note. This raises the question of the desirability of a revision of certain unnecessarily prolonged cooking processes used in the commercial preparation of certain fruit, vegetable and cereal products.

SUMMARY

1. Grapefruit juice, fresh and concentrated was found to furnish very small amounts of vitamin A when tested on young rats reduced to a state of vitamin A deficiency.

2. Pressed grapefruit oil and the ether extract of grapefruit peel were ineffective under like conditions.

3. Lemon juice likewise seemed to be of little value as source of vitamin A although because of difficulty in inducing the rats to take this juice some doubt is felt as to the degree of failure ascribable to the nature of the juice.

4. The ether extract of lemon peel was found to exert a small but definite curative effect upon vitamin A deficiency. This is comparable with the action of dried lemon peel previously reported.

5. The striking contrast afforded by the high vitamin A content of orange juice, peel and oil with the low content of the corresponding parts of the lemon and grapefruit is offered as an example of the futility of attempting to predict vitamin values from botanical or chemical relations. A more orderly relation between character of lipochromes in these fruits and their vitamin A content is postulated.

6. The value as sources of vitamins A and B of samples of commercial and homemade orange and lemon marmalades and candied orange peels was found to differ considerably, the homemade products showing a considerably higher value in all cases than did the commercial products. This is apparently an illustration of the possibility of controlling vitamin destruction by modifications of certain cooking processes in favor of shorter periods and lower temperatures.

BIBLIOGRAPHY

(1) Morgan: This Journal, 1923, lxiv, 522.
(2) Osborne and Mendel: Journ. Biol. Chemistry, 1920, xlii, 465.
(3) Osborne and Mendel: Proc. Soc. Exper. Biol. and Med., 1922, xix, 187.
(4) Davey: Biochem. Journ., 1921, xv, 83.
(5) Chick, Hume and Skelton: Lancet, 1918, ii, 735.

THE AWAKENING OF COPULATORY ABILITY IN THE MALE ALBINO RAT[1]

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1. Purpose of investigation. Published reports on the age of pubescence in the male rat reveal a range of variation somewhat greater than that commonly ascribed to errors of sampling and individual differences. One suspects that differences in the technique of rearing and testing the males account for a large part of this variation. For this reason comparative studies are especially needed in order that sources of discrepancy may be ascertained and ways for their correction be suggested.

The object of the present investigation is *1*, to determine the age at which copulatory ability normally awakens in the male albino rat reared and tested under optimum conditions of nutrition and environment; and *2*, to correlate the awakening of copulatory ability with the maturation of spermatozoa and the development of the organs of intromission.

2. Animals used, housing and diet. The young males used in this study were born of adult parents approximately one year of age and were taken from thirty consecutive litters[2] delivered within a period of 10 days. From the age of weaning (20 days) they were housed in wire mesh cages (dimensions 22 × 18 × 8 inches) resting on a bed of fine wood shavings. Parents and offspring were given a staple diet consisting of the following food ingredients thoroughly mixed.

Standard diet. I (McCollum diet)

Whole wheat, ground fine	67.5
Casein (80 mesh)	15.0
Whole milk powder	10.0
Sodium chloride	1.0
Calcium carbonate	1.5
Butter fat	5.0
	100.0

[1] This investigation was financed by a grant from The Committee for Research on Sex Problems of the National Research Council.

[2] This group constitutes one-half of the males reared from thirty consecutive litters. At the age of weaning the males of these litters were *paired according to body weight*. One animal of the pair served as a control for the other which was placed on a deficiency diet. The controls make up the group of fifty-six animals herein considered. Results pertaining to the animals placed upon deficiency diets will be published in a subsequent paper.

The parents were maintained upon this diet for a period of three months prior to the date of breeding for the present study. Food in excess of the amount daily consumed was placed into the cages. Thus the food intake, with respect to time and quantity, was wholly regulated by the dictates of appetite. Pollution of food and water by excrement and cage bedding was prevented insofar as possible by the use of receptacles especially adapted to this end.

3. Tests for copulatory ability. The term "copulatory act" is herein employed to denote the overt elements of the copulatory response without implication as to the delivery of spermatozoa into the reproductive tract of the female. In other words, it refers to those elements of the reproductive act that are readily discerned by the observer without the aid of instruments. For purposes of description, it may be resolved into the following elements:[3]

a. Pursuit and mounting.

b. Palpation of the female's sides.

c. On the part of the female, depression of the lumbar region of the back with consequent elevation of the sacro-coccygeal region.

d. Pelvic movements of the male by which the penis is thrust toward the vagina in piston-like manner.

e. Cessation of palpation of female's sides and the backward lunge.

f. Licking of the penis.

The copulatory act is ofttimes performed by adult males without intromission. More frequently, however, is this to be observed in young males of small stature or young males with organs of intromission only partially developed.

Tests for copulatory ability in young males were made in the following manner. A sexually receptive female was introduced into the cage of a young male or pair of males. There, under the observation of the experimenter, she was confined for a period of eight or ten minutes. If no signs of sexual activity were manifested by the young male in that period of time she was removed to the cage of another male and similarly observed for eight or ten minutes. After a lapse of from one-quarter to one-half hour the female was again returned to the cage of the young male first tested for another period of eight minutes' duration. If in the second period no copulation was observed still another period of testing was allowed from one-quarter to a half-hour later. Thus each evening (in some cases alternate evenings) the young male was given three opportunities to manifest copulatory ability. When possible a different female was placed with each male for each of the

[3] This topic is discussed in considerable detail in an earlier article by the author entitled "The Congenital Sexual Behavior of the Young Male Albino Rat," 1922.

three periods. Shifting of females tends to prevent any one male being confined for more than one period with a relatively inactive or unresponsive female.

Practical experience with a large number of males under various conditions of cage adaptation has led the experimenter to proceed upon the assumption that if a young male, thoroughly tame and cage-adapted, does not show signs of sexual excitement in the presence of receptive females during the first eight or ten minutes of an observation period, providing he had thoroughly explored the female during that time, he will not ordinarily show signs of sexual excitement in the remainder of a period lasting over one-half hour or more. Exceptions to this practical rule have sometimes been observed in the course of routine testing, but their frequency has always been low. Furthermore, a young male, on the threshold of puberty, that is slow in making the initial copulatory response will a day or two later make this response almost immediately after the female is placed into the cage. Thus even in these exceptional cases the first copulatory act will be delayed only a day or two at most when nightly observations are in progress. This delay, being both infrequent and small in amount, would not appreciably alter the measures of central tendency and variability given in this paper.

A colony of 150 to 200 sexually mature females was maintained during the time young males were being tested for copulatory ability to insure an adequate supply of receptive animals for each night's work. Any female that showed a disposition to attack young males was immediately discarded lest her repeated attacks upon the latter might establish fear responses of sufficient strength to prevent their further exploratory activities.

THE AWAKENING OF COPULATORY ABILITY. Experimenters who have dealt with the age of pubescence in male rats have not published the raw data on which their statements concerning the first manifestations of copulatory ability are based. For this reason comparative studies on this point cannot be made at the present time, however desirable they may be. Steinach (13), in 1894, reported that young males become sexually active when about ninety days of age. Hewer (5) states that the male begins copulation by the tenth week (70 days). Observations by different investigators (Jackson (6) and others) working in this country under well standardized laboratory conditions indicate that seventy to eighty days is the common age for the manifestation of copulatory ability. Individual cases of precocious copulation have been reported by various workers. Kirkham (8) gives one instance of a male impregnating a female at the early age of 57 days. Stone (14) and Slonaker (12) each recorded a case of copulation at the age of 63 days.

1. Results from this study. The ages at which fifty-six young males first copulated constitute the basis of the tentative norms herein given. At the outset an effort was made to bring into the study a group of

TABLE 1

The weights of 56 young males at weaning time (age, 20 days)

	WEIGHT IN GRAMS							
	24–25	26–27	28–29	30–31	32–33	34–35	36–37	Total
Frequency	1	6	8	19	10	6	6	56
Per cent	1.79	10.72	14.29	33.93	17.86	10.72	10.72	100

Mean 31.61 M.V. 2.17 Median 31 (ungrouped data) M.V. 2.19

young males that might be taken as representative of the colony as a whole. The degree to which this ideal was approached may be inferred from a consideration of the distribution of weights of these fifty-six animals at weaning time.

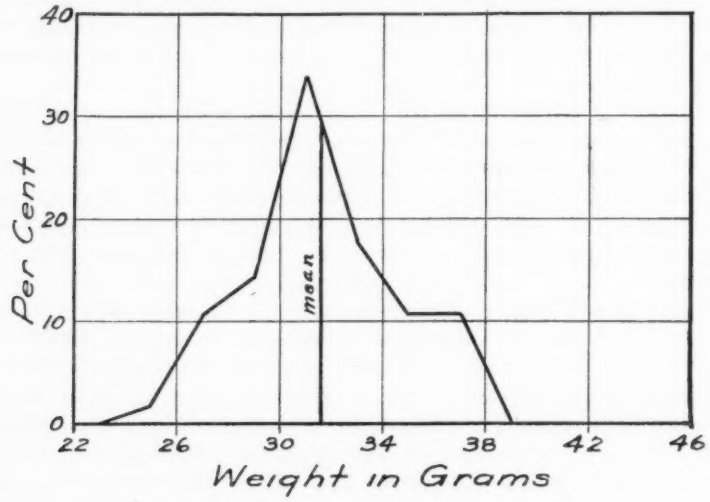

Fig. 1. Frequency polygon plotted from the data of table 1 showing the distribution of weights of the fifty-six young males used in this study.

a. Early development: When twenty days of age the fifty-six males were weaned. Their weights at this time are given in table 1. The weights range from 25 to 36 grams. The mean weight is 31.6 (M. V. 2.17) and the median (ungrouped data) is 31 grams (M. V. 2.19).

A frequency polygon plotted from the data of table 1 is shown in figure 1. The form of this curve roughly approximates that of the probability curve of a normal distribution. Thus, it would seem, the weights of the animals of this group are distributed in much the same manner as would be found in any random selection of cases and is therefore likely to be fairly representative of the colony from which it was taken.

TABLE 2

Comparative data on the mean body weight of animals of the present study and similar weights for representative groups of animals from other laboratories

INVESTIGATOR	NUMBER OF CASES	AGE	MEAN WEIGHT	LOW	HIGH	DIET
Donaldson (2)	19	21	21.2	14.6	30.6	Bread and milk; whole corn, varied with vegetables, meat and sunflower seed
Stone	56	21	32.3	27.0	38.0	Standard diet no. 1
Jackson (6)	24	22	22.2	13.5	38.0	Bread and milk; chopped corn; meat once a week
Stone	56	22	34.2	28.0	41.0	Standard diet no. 1
King (7)	50	30	48.5	41.0	60.0	Table scraps; whole corn
Stone	56	30	55.9	42.0	67.0	Standard diet no. 1
Slonaker (11)	58	20	20.56	15.0*	26.0*	Table scraps
Stone	56	20	31.6	25.0	37.0	Standard diet no. 1
Evans (3)	535	30	50.0	40.0	75.0	Standard diet no. 1
Stone	56	30	55.9	42.0	67.0	Standard diet no. 1

* Average of weights of males of lightest and heaviest litters. Two males in each litter.

In order that the early growth of these males may be compared with that of animals reared in other laboratories, under somewhat different conditions of housing and diet, the data of table 2 have been brought together. The publications from which these data were taken do not give weights at any one age. For this reason it seemed advisable to give the weight for my group at each of the ages selected to represent the animals of other workers. Thus in each case a direct comparison may be made.

As the foregoing data show, my animals as a group are generally heavier than those from other laboratories. Evans' weights for females roughly approximates that of my own as might have been forecast since the two groups were fed upon the same diet. The group of King,

TABLE 3

Mean weight of the group of fifty-six animals of this study from the age of weaning at 20 days to the date when the last animal of the group was sacrificed

AGE	NUMBER OF CASES	MEAN WEIGHT	AGE	NUMBER OF CASES	MEAN WEIGHT
20	56	31.1	50	22	125.4
21	56	32.3	51	19	127.9
22	56	34.2	52	17	129.7
23	56	36.9	53	15	134.5
24	56	39.8	54	13	136.5
25	56	42.6	55	12	141.4
26	56	45.1	56	10	142.4
27	56	47.8	57	6	147.7
28	56	50.1	58	5	152.0
29	56	52.7	59	5	155.0
30	56	55.9	60	4	155.7
31	56	59.3	61	4	159.0
32	56	62.8	62	3	160.3
33	56	66.3	63	3	164.0
34	56	69.9	64	3	168.0
35	56	73.7	65	3	171.3
36	56	77.2	66	3	174.9
37	56	81.0	67	2	189.0
38	55	84.7	68	1	174.0
39	55	88.7	69	1	176.0
40	55	92.7	70	1	179.0
41	49	95.3	71	1	182.0
42	39	96.6	72	1	185.0
43	36	100.6			
44	35	104.3			
45	34	108.0			
46	32	111.4			
47	30	114.8			
48	25	117.8			
49	22	122.8			

TABLE 4

The ages at which the initial copulatory act was performed by fifty-six animals herein studied

	AGE IN DAYS							
	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74
Frequency	1	21	12	10	8	1	2	1
Per cent	1.79	37.5	21.43	17.86	14.28	1.79	3.57	1.79

which next approaches my own, is not strictly comparable to mine since, according to her own statement, the animals of her group represent the best of the colony and are not to be regarded as representative of the Wistar colony at that time. My own group, on the other hand, is representative of the colony. Although the animals are large for their ages, their weights are only normal for animals grown under a superior dietary regime.

The mean weights for the group of fifty-six animals from the age of weaning at 20 days to the date when the last animal of the group was sacrificed are given in table 3. The total number of the group is represented in these averages up to the 37th day. Thereafter the number

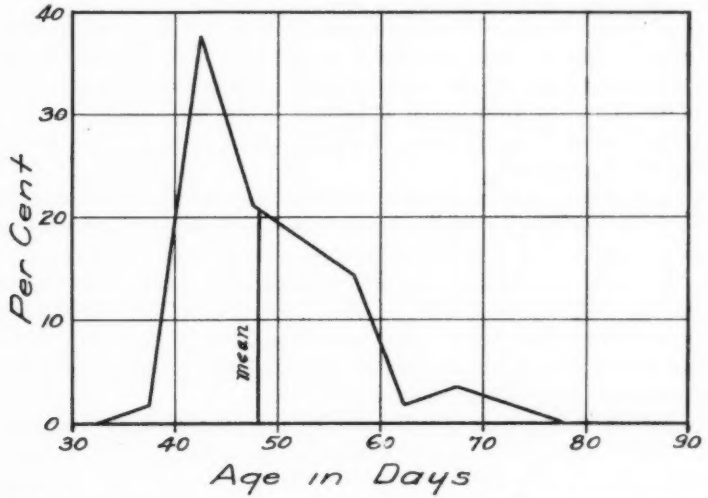

Fig. 2. Frequency polygon plotted from the data of table 4 showing the distribution of ages at which the initial copulatory act was performed by fifty-six animals herein described.

begins to dwindle owing to the practice of sacrificing each animal immediately after the initial copulation in order that the status of development of the reproductive tract might be ascertained.

b. Mean age of copulation: The age at which the initial copulatory act was performed by each of the fifty-six animals is given in table 4. These ages range from 37 days to 72 days. The mean of the distribution is 48.09 days (M. V. 5.93) and the median (ungrouped data) is 47 (M. V. 6.09) days.

Figure 2 gives the form of the frequency polygon which results when the data of table 4 are graphically represented.

One outstanding feature of this distribution is the concentration of cases at the lower age limits. The mode falls at the age of 41 or 42 days. Somewhat over one-third of the entire group copulated prior to the 43rd day of age. The technique of testing animals for copulation may have been responsible for a small amount of the massing of cases at the ages of 41 and 42 days, yet it is not responsible for the *general* shunting of cases toward the earlier ages. The original plan[4] of testing called for the first test to be given at the age of 40 days. When, however, nineteen young animals copulated on the 40th and 41st day of age, it seemed advisable to give earlier tests, insofar as it was possible, to the remaining animals that had not yet attained the age of 40 days. Approximately ten animals were then tested from the age of 35 days. One of these copulated at the age of 37 days. It is quite reasonable to suspect that of those copulating at the age of 40 days some might have copulated one or two days earlier if opportunity had been given. This would have thrown a few cases now in the class group of 40 to 44 days to that of 35 to 39 days, thereby making the ascent of the curve somewhat less abrupt.

It is a point of some practical interest to note that the weights of animals at the time they first manifest copulatory ability vary over a wide range. The weights of the young animals of this group at the time of initial copulations vary from 90 grams to 200 grams. In figure 3 the age-weight relationship at the time of copulation is shown for each animal of the group. The mean weight of all animals remaining in the distribution from the 37th to the 72nd day when the last animal was sacrificed is shown by the solid line.

Referring to the dots which indicate the weight of each animal at the time of copulation one may note that the earliest cases of copulation occurred among rats that were, for the most part, well above the average in weight. After the 42nd day, however, the number of animals sacrificed with weights below the average approximately equals the number with weights above the average.

A mathematical expression of the relationship between body weight and age at the time of copulation has been secured by calculating the coefficient of correlation between these two variables. A coefficient (Pearson) of 0.242, P.E. 0.068 was found. Although positive, this coefficient is very low and, taken in relation to its probable error, indicates little predictability of copulatory age on the basis of body weight.

c. The copulatory act of the young male contrasted with that of the adult: Minor dissimilarities between the pattern of the copulatory act of the

[4] Copulation at the early age of 40 days was wholly unexpected by the experimenter. The earliest previous record for copulation was 57 days, and the average was thought to fall between 70 and 80 days.

young and the adult male may be observed. For the most part the cause of these is to be attributed to limitations of physical vigor and stature. Some, on the other hand, appear to be due to lack of sexual experience, i.e., to incomplete coördination of the elements of the sexual apparatus. Since these topics have been discussed at some length in a previous paper, (14), however, the details need not be repeated.

THE RELATION BETWEEN THE AGE OF COPULATION, MATURATION OF SPERMATOZOA AND DEVELOPMENT OF THE ORGANS OF INTROMISSION. The male's ability to inseminate the female implies development along three lines: *1*, the awakening of copulatory ability; *2*, the maturation of spermatozoa; and *3*, the development of the organs of intromission.

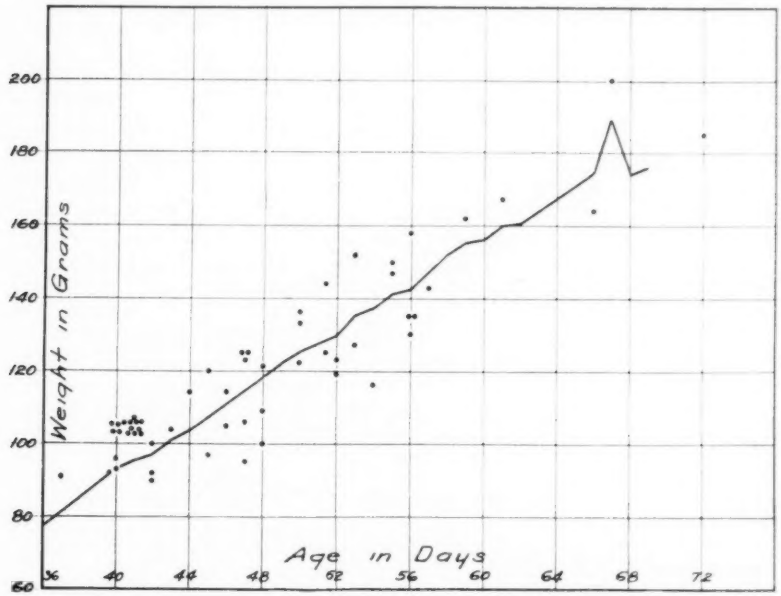

Fig. 3. Age-weight relationship of fifty-six males at the time of the first copulation.

Although development along these three lines is simultaneous the rates vary to such a degree that any one may get out of step with the others. Since ability to beget young is dependent upon all of the three factors, the age of sexual maturity will thus be retarded in accordance with the most retarded of the three. For example, a young male may be able to perform the overt copulatory act, but have no spermatozoa to deliver to the female. Or, a male may have spermatozoa in great numbers, but be sexually inactive in the presence of the female. Yet again, he may be sexually active, have spermatozoa in great numbers in the reproductive tract, but

because of under-developed organs of intromission be unable to deliver the spermatozoa into their proper receptacle in the tract of the female. Cases illustrating each of these possibilities have been encountered in the study of these fifty-six cases.

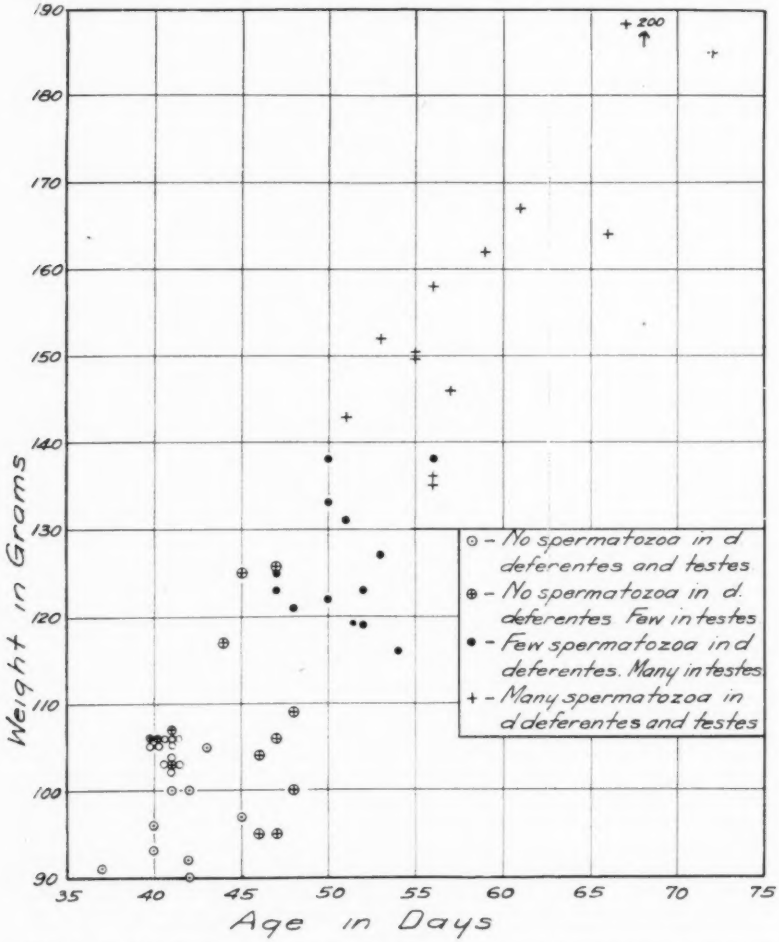

Fig. 4. In this figure data bearing on the maturation of spermatozoa in relation to the age of copulation and the body weight are given. Fifty-six cases.

1. The maturation of spermatozoa in relation to the initial copulatory act. Norms for the maturation of spermatozoa in rats reared under conditions

similar to those of this study are not available. Various investigators have published results on this point for animals grown at a slower rate, but distribution tables for these likewise are not available for comparison. Allen (1) reports thirty-seven days as the earliest age at which spermatozoa appeared in the lumina of the seminiferous tubules. His animals were taken from the Wistar colony. Siperstein (10), working in the Institute of Anatomy, University of Minnesota, found them present at the age of 43 days. Hewer (5) states that spermatozoa were present in the tract as a general rule at the age of sixty days. Donaldson (2) likewise reports that sixty days conforms to the age of sexual maturity of rats as determined by histological methods. Since the earliest age, prior to the present study,

TABLE 5

The maturation of spermatozoa in relation to the age at which the initial copulatory act was performed by the fifty-six animals of this study

(Read columns from top to bottom)

STATUS OF SPERMATOZOA	AGE IN DAYS				
	Prior to 45 days	45–49	50–54	55–59	60 and up
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
No spermatozoa in ductus deferentes and testes	77.3	8.3	0	0	0
No spermatozoa in ductus deferentes, few in testes	22.7	66.6	0	0	0
Few spermatozoa in ductus deferentes, many in testes	0	25.0	80.0	12.5	0
Many spermatozoa in ductus deferentes and testes	0	0	20.0	87.5	100.0
Number of cases	22	12	10	8	4

at which the male had been known to copulate was 57 days and the average was thought to fall above 70 days one should have concluded that spermatozoa antedate by ten or twenty days the initial copulatory act. Such a conclusion, however, cannot be drawn from the results of this study.

Each of the fifty-six animals was sacrificed a few hours after the initial copulatory performance. The testes, together with the ductus deferentes were removed for study. A drop of spermatic fluid expressed from each ductus deferens into a drop of normal saline was examined microscopically to determine whether spermatozoa had reached this part of the reproductive tract. Also portions of the testes, macerated so as to break up the seminiferous tubules and thus permit egress of free moving spermatozoa, were likewise subjected to microscopic examinations. In each case notations were made as to whether spermatozoa were present, active or inactive, and whether rare or plentiful.

The data bearing upon the maturation of spermatozoa gathered in these routine examinations are brought together in figure 4.

On this chart each animal is represented by one of four simbols, viz.: a circle, a cross enclosed by a circle, a solid dot or a cross. These symbols indicate the status of the spermatozoa in the reproductive tract; their heights on the ordinate give the animals' weights and their positions on the abscissa, the ages in days.

To facilitate reading the results from this chart a table indicating the per cent of animals falling into each of the categories designated by the symbols used on the chart is given below in table 5.

As this chart shows, the rats copulating at the extremely early ages had only a few or no free spermatozoa in either the testes or the ductus deferentes; those copulating at the latest ages had many active spermatozoa in both ductus deferentes and testes; and those falling between these extremes represent intermediate states. At the ear'ier ages spermatozoa, in small numbers, are found first in the testes; a few days later they are more numerous in the testes and some will by that time be found in the ductus deferentes. Still later the number seen in the testes is much greater and likewise that in the ductus deferentes will have greatly multiplied. Thus, in a general way, the results are in harmony with the results of Allen (1) and Siperstein (10) which indicate that the number of spermatozoa found in the testes or the reproductive tract increases rapidly after the first crop of spermatozoa appear.

A fact of both theoretical and practical interest brought out by the foregoing data seems worthy of further elaboration and emphasis. In this group of young males a considerable degree of overlapping between the age of awakening of copulatory ability and the ripening of the first crop of spermatozoa occurs, yet it would be manifestly unsafe, where accurate knowledge of the awakening of copulatory ability is desired, to assume its presence from a knowledge that spermatozoa are in the lumina of the seminiferous tubules or even in other receptacles of the reproductive tract. Of the fifty-six cases herein considered, nineteen copulated *before* free moving spermatozoa were found in the testes. On the other hand the remaining thirty-five cases, all of which had spermatozoa in the testes, undoubtedly had them there from one to twenty or more days prior to copulation. With such lack of conformity between the awakening of copulatory ability and the appearance of the first crop of spermatozoa *neither of these functions can be looked upon as an unequivocal criterion of the presence of the other.*

To find a degree of independence in the awakening of copulatory ability and the maturation of spermatoza is not wholly unexpected. It is a fact long since demonstrated that there is not a dependent relationship between the germinal tissue and the behavior aspects of the sexual functions. Now it appears doubtful whether they are even dependent upon the same factor.

2. Development of the organs of intromission. Immaturity of the organs of intromission accounts primarily for the inability of young animals to

inseminate females in the earlier stages of puberty, although they manifest copulatory ability and have mature spermatozoa. Previous studies have shown that the coagulatory glands and seminal vesicles are not necessary for insemination of the female rat.[5] This being the case, there is no reason to suspect that immaturity of these glands prevents the insemination of females by the younger males. To be sure the number of impregnations may be greatly reduced[5] if the glands are underdeveloped, but they are not to be regarded as the *sine qua non* of impregnation.

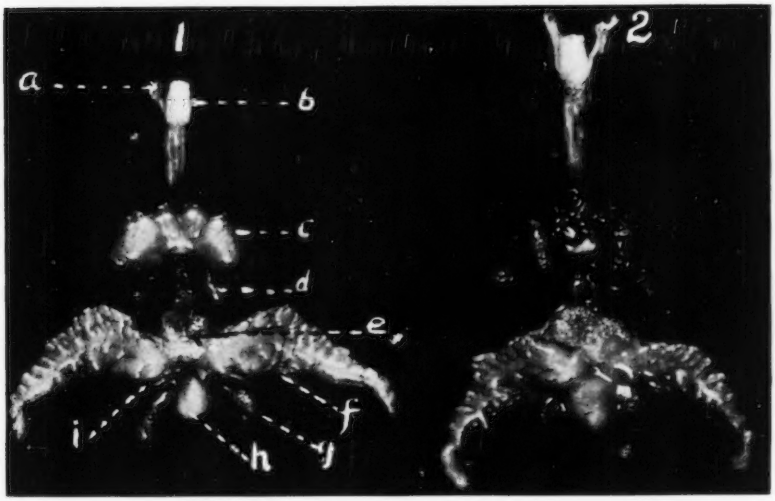

Plate 1. The names of structures labeled for animal 1 are as follows: *a*, prepuce; *b*, glans penis; *c*, muscle; *d*, Cowper's gland; *e*, prostate gland; *f*, seminal vesicle; *g*, coagulatory gland; *h*, urinary bladder; *i*, stub of ductus deferens.

The penis of rats copulating at the age of 40 to 50 days is very small. The cartilage core is not yet covered by the glans penis and the organ cannot be everted from its sheath. Between the ages of 50 and 60 days its length materially increases and the glans grows out over the cartilage. Toward the age of 55 days the penis of precocious males becomes eversible and is then, under extremely favorable conditions, of sufficient length to reach the vaginal orifice of the female. At this age, therefore, those males which have spermatozoa in the reproductive tract, and can execute the copulatory act may, occasionally, inseminate the receptive female.

The following photographs roughly illustrate the development of the acessory reproductive apparatus at maturity, and in the early ages of

[5] For details on this point see Marshall, *The Physiology of Reproduction*.

puberty. With no. 1 a description of the chief elements of the accessory sexual apparatus herein featured is given. Similar structures may be readily identified in the other figures.

The sexual apparatus of nos. 1 and 2 were taken from adult males. That of no. 1 came from a male 170 days of age that weighed 256 grams. Although his maximum growth had not yet been attained he may be regarded as typical of the young adult male. The sexual organs, likewise, had not completed their growth as may be inferred from a consideration of no. 2. The apparatus of no. 2 came from a male approximately 2½ years old and weighing 385 grams. Both 1 and 2 were sires of litters and, at the time of sacrifice, were sexually aggressive. They fairly represent the stage of development toward which each of the reproductive tracts pictured below was approaching.

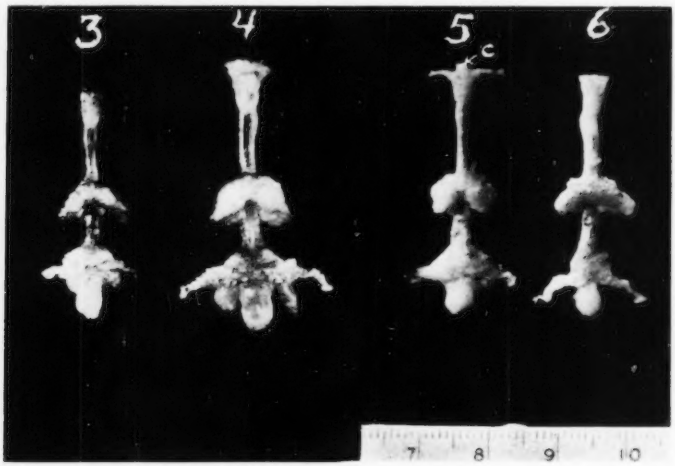

Plate 2

On plate 2 the sexual organs of two precocious males, aged 40 and 55 days, are shown (nos. 3 and 4 at left). Both males copulated at the early age of 40 days. At the time of sacrifice no. 3 weighed 105 grams (above the average). No spermatozoa were found in either the ductus deferentes or the testes. Animal no. 4 was not sacrificed on the day of the initial copulation. He was placed with a receptive female on alternate nights after the first copulation and allowed to copulate at will during the night. Microscopic examinations of smears from the vagina of the female were made after twenty or thirty copulations to determine whether spermatozoa had been delivered into the tract of the female. Also the female with which

the male had cohabited during the night was isolated for a period of three weeks to ascertain whether conception resulted. When fifty-five days of age this male delivered spermatozoa into the reproductive tract of a female. This female conceived and bore a litter. The male was sacrificed and examined on the following morning. The penis was found eversible and the cartilage well covered by the glans penis. Active spermatozoa in great numbers were present in both the ductus deferentes and the testes. In every way, therefore, this young male is qualified to represent the type of precocious, sexually mature male rat in the early stages of puberty. *In him the sexual libido had awakened; spermatozoa had matured; and the organs of intromission were sufficiently developed to deliver spermatozoa into the tract of the female.*

The organs pictured at the right on plate 2 illustrate the development of sexual apparatus in the average young male at the age of 55 days. Males 5 and 6 developed at a rate corresponding to that of the average case in the group of fifty-six animals previously discussed. They copulated at the age of 45 days, but were unable to inseminate females at the age of 55 days, when sacrificed. Active spermatozoa were found in the tract of each animal, but the organs of intromission were apparently not sufficiently developed to enable the males to deliver spermatozoa. The cartilage was only partially covered by the incompletely developed glans. This condition is shown in the case of no. 5 where the foreskin has been partially removed so as to expose the cartilage, *c*. Animals 5 and 6 weighed at the time of sacrifice 133 and 140 grams respectively (approximately average weight for the group of fifty-six rats at this age).

Although adequate records are not available to indicate the age at which rats developing at the rate of these two cases become *sexually mature*, some light on this problem is thrown by data from five other cases typifying the average animal. These five animals copulated for the first time between the ages of 45 and 48 days. At the age of 65 days each of these males inseminated a receptive female. No tests for ability to inseminate females prior to that age were given. On plate 3 the sexual apparatus of two of these males, nos. 7 and 8, removed at the age of 65 days, are given.

Contrast the development of the various organs featured in nos. 7 and 8 with similar organs shown in nos. 5 and 6. No. 7 in which the glans penis is completely exposed illustrates the manner in which the cartilage core of the penis becomes hooded by the glans. In neither case are the seminal vesicles well developed, but as has been heretofore demonstrated, their development or even their presence, is not essential to impregnation.

The tract of no. 9 is of interest because it illustrates the fact that age is a factor in the development of the sexual apparatus as well as the general nutritional factors which condition bodily growth. No. 9 was sacrificed at the age of 90 days; body weight was 156 grams, approximately the same

as that of nos. 7 and 8 which were sacrificed when 65 days of age. Obviously his bodily development was much slower than that of the animals discussed in this study. He copulated at the age of 75 days. Note that the reproductive tract at the age of ninety days is in an excellent state of development; the glands are turgid with secretory products. The individual organs, although small for a rat of ninety days, are well formed and closely resemble those of the adults featured on plate 1.

3. The age of sexual maturity. Because of discrepancies between the awakening of copulatory ability, the maturation of spermatozoa, and the development of organs of intromission it seems obvious that the age of sexual maturity can be accurately determined only by requiring the young male to demonstrate ability to inseminate the receptive female. Data from the present study of fifty-six young males are not sufficiently complete

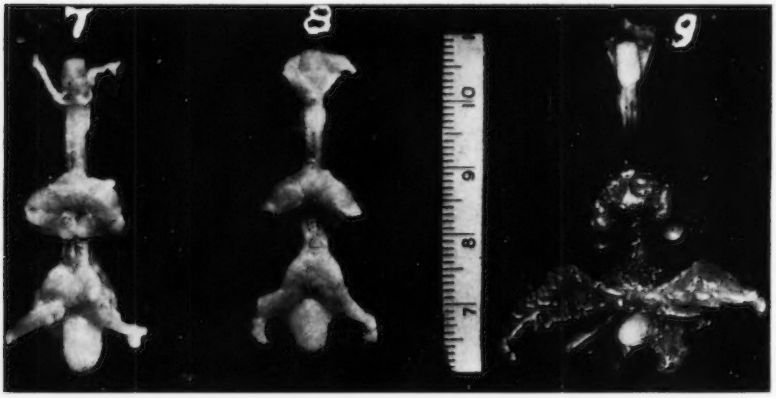

Plate 3

to enable one accurately to determine the mean age of sexual maturity. Only a rough approximation can be suggested from the results at hand. It probably falls at or near the age of 65 days. As has been heretofore stated, the mean age when copulatory ability was first manifested is 48.09 days. All animals copulating after the age of 45 days, and five before, had mature spermatozoa in the testes (see fig. 4). Furthermore, few cases have been discovered in routine examinations at the ages of 60 to 70 days of over forty animals similarly reared, wherein the penis was not as well developed as those illustrated by animal 4, plate 1, and animals 7 and 8, plate 3. On the basis of these data it would seem that the age of 65 days is a conservative estimate of the age of sexual maturity for rats such as the group herein considered. The earliest age accurately determined in connection with

this and other studies was 55 days; the latest, 153 days. Extreme variations from the mean age will undoubtedly be greater in the upper age limits than in the lower limits because of the greater possibilities for variation in that direction.

CONCLUSIONS

1. The mean age at which copulatory ability was first manifested by fifty-six male rats reared under optimum conditions of nutrition and environment was 48.09 days (M. V. 5.93 days); the median age, 47 days (M. V. 6.09 days). Ages ranged from 37 to 72 days. One third of the group copulated prior to the age of 43 days.

2. Body weights of the fifty-six animals at the time of the initial copulatory act range from 90 to 200 grams. Although the earliest cases of copulation occurred among rats that were, for the most part, well above the average in body weight, the coefficient of correlation between body weight and age at the time of copulation was only 0.242, P. E. 0.068, for the entire group.

3. The male's ability to inseminate a female implies development along three lines: *1*, the awakening of copulatory ability; *2*, the maturation of spermatozoa; and *3*, the development of the organs of intromission. Although simultaneous development along these three lines normally occurs in the young male, either of them may be accelerated or retarded with respect to the others. The age of sexual maturity is retarded in accordance with the most retarded of the three factors.

4. Nineteen of the fifty-six males copulated before mature spermatozoa had been liberated in the tubules of the testes. The oldest animal without spermatozoa in the testes was 45 days of age. The youngest animal with spermatozoa in the testes at the time of copulation was 40 days old. Of thirty-four animals copulating at or after the age of 45 days only one was found without spermatozoa in the testes (aged 45 days). At the age of 50 days and thereafter spermatozoa were plentiful in the testes and ductus deferentes.

5. In animals copulating at the earlier ages (below 55 days) the organs of intromission were not sufficiently well developed to enable males with spermatozoa in the reproductive tract to inseminate females. One animal of this group inseminated a female at the early age of 55 days. The mean age at which males grown similar to this group can accomplish the act of insemination under favorable circumstances, probably falls at or near 65 days.

BIBLIOGRAPHY

(1) Allen: Anat. Rec., 1919, xvi, 93.
(2) Donaldson: The rat, 1915.
(3) Evans and Bishop: Journ. Metab. Res., 1922, xx, 319.
(4) Long and Evans: Memoirs of the Univ. of Cal., 1922, vi.
(5) Hewer: Journ. Physiol., xlvii, 479.
(6) Jackson: Amer. Journ. Anat., 1913, xv, 1.
(7) King: Anat. Rec., 1915, ix, 403.
(8) Kirkham: Amer. Jour. Anat., 1913, xv, 291.
(9) Marshall: The physiology of reproduction. London, 1910.
(10) Siperstein: Anat. Rec., 1921, xx, no. 4.
(11) Slonaker and Card: This Journal, 1923, lxii, 503.
(12) Slonaker and Card: This Journal, 1923, lxiv, 35.
(13) Steinach: Arch. f. d. gesammt. Physiol., 1894, lvi, 95.
(14) Stone: Journ. Comp. Psychol., 1922, ii, 95.